Induction and characterization of endotoxin tolerance in equine peripheral blood mononuclear cells *in vitro*

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ABSTRACT

Endotoxemia is responsible for severe illness in horses. Individuals can become unresponsive to the endotoxin molecule after an initial exposure; this phenomenon has been called developing a state of 'endotoxin tolerance' (ET). ET has been induced in horses *in vivo*; however, cytokine expression associated with ET has not been investigated. The purpose of this study was to develop and validate a method for inducing ET in equine peripheral blood mononuclear cells (PBMCs) *in vitro*, and to describe the cytokine profile which is associated with the ET.

Blood was collected from 6 healthy horses and PBMCs were isolated. ET was induced by culturing cells with three concentrations of endotoxin given to induce ET, and evaluated after a second dose of endotoxin given to challenge the cells. The relative mRNA expression of IL-10 and IL-12 was measured by use of quantitative PCR.

ET was induced in all cells (n=6) exposed to the 2-step endotoxin challenge. In PBMCs treated with 1.0 ng/ml of endotoxin followed by challenge with 10 ng/ml of endotoxin, the relative mRNA expression of IL-10 in tolerized cells was not different from positive control cells. In contrast, the relative mRNA expression of IL-12 in tolerized cells was decreased by 15-fold after the second endotoxin challenge compared with positive control cells.

This experiment demonstrated a reliable method for the *ex vivo* induction of ET in equine PBMCs. A marked suppression of IL-12 production is associated with ET. The production of IL-10 was not altered in ET in our model.

DEDICATION

To my parents, Iris and Albert Frellstedt,

and Nancy Beth Anderson.

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LIST OF ABBREVIATIONS

AP-1	activator protein-1
APCs	antigen-presenting cells
BPI	bactericidal/permeability increasing protein
bwt	bodyweight
CD14	cluster of differentiation no. 14
cDNA	complementary deoxyribonucleic acid
DCs	dendritic cells
ELISA	enzyme-linked immunosorbent assay
ET	endotoxin tolerance
HDL	high-density lipoprotein
IL	interleukin
IRAK1	interleukin-1 receptor-associated kinase 1
IRAK4	interleukin-1 receptor-associated kinase 4
kDa	kilodaltons
kg	kilograms
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharides
MAPK	mitogen-activating preotein kinase
mCD14	membrane-bound cluster of differentiation no. 14
MyD88	myeloid differentiation primary response protein 88
NK	natural killer
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinases

RNA	ribonucleic acid
sCD14	soluble cluster of differentiation no. 14
TAK1	TGF-beta activated kinase 1
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor β
Th	T helper
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF-α	tumor necrosis factor α
TRAF6	TNF receptor-associated factor 6

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Chapter 1. Endotoxemia in horses

Endotoxin

Endotoxins (lipopolysaccharides, LPS) are unique glycolipids that constitute a major portion of the outer leaflet of the outer membrane that is unique to gram negative bacteria (Schultz and Weiss 2007). Endotoxins consist of a highly conserved lipid A region, a core polysaccharide region and a repeating tetra- or penta-saccharide that constitutes the O-antigen. Endotoxins induce potent responses in a wide range of host species including humans by rapidly inducing cellular biosynthesis and release of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) and other bioactive metabolites and secondarily by rapidly activating extracellular complement, clotting, and fibrinolytic pathways. Local responses to endotoxin in tissue generally involve protective inflammatory host-defense cascades but more profound responses at the systemic level can lead to a pathologic state ranging from fever to the fulminant sepsis-syndrome with multiorgan failure and high mortality (Schultz and Weiss 2007).

The lipid A moiety is highly conserved among gram-negative bacteria, and is the component of lipopolysaccharide which binds to LPS-binding protein (LBP) in the plasma, initiating a cascade of events which results in endotoxic shock (see Figure 1). This is mediated by a complex series of biochemical processes, initiated by cellular binding, transmembrane signaling, activation of cytokine expression leading to systemic inflammation and circulatory shock. Once LPS-LBP complexes are bound to Cluster of Differentiation antigen 14 (CD14) on the surface of phagocytes, CD14 interacts with Toll-like receptor 4 (TLR-4) resulting in activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways and subsequent production of pro-inflammatory (TNF-α, IL-1, IL-6, IL-8, IL-12) and

anti-inflammatory (IL-10, TGF-β) cytokines (Schutt 1999; Werners *et al.* 2005). TNF-α represents the most important and most potent pro-inflammatory mediator involved in endotoxic shock. The pathophysiologic events of gram-negative septic shock are mediated by the endotoxin-induced systemic overproduction of TNF-α. TNF-α acts alone and by the induction of IL-1 and numerous more distal mediators, leading to potentially lethal multiorgan tissue damage (Robinson *et al.* 1993).

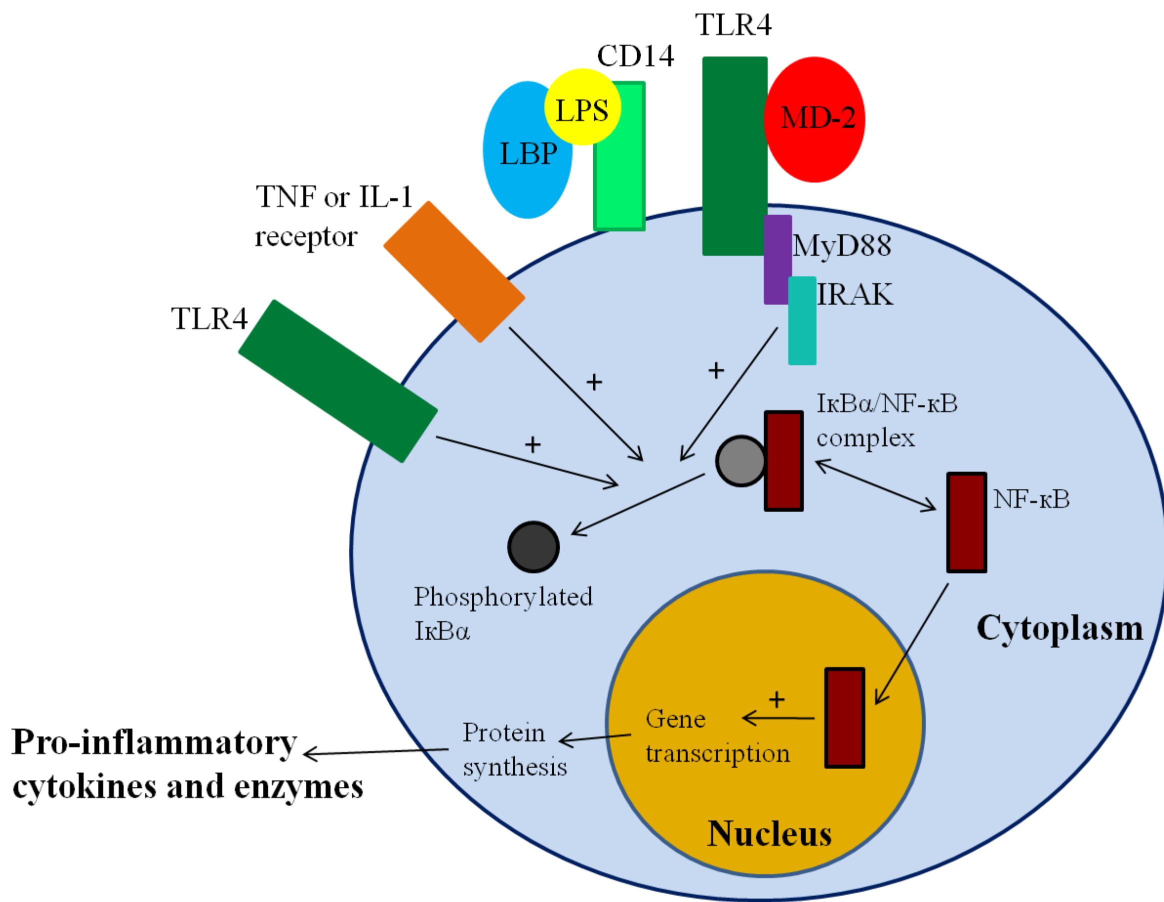

Figure 1. Biochemical cascade initiated by LPS and other pro-inflammatory agents leading to the production of pro-inflammatory cytokines. The transcription activator nuclear factor (NF)-κB is normally retained in the cytoplasm in an inactivated state, preventing the unregulated production of inflammatory mediators. (Used with permission of H.C. McKenzie III, 2010)

Endotoxemia in horses

Endotoxemia is defined as the presence of endotoxin (LPS, lipopolysaccharide), which is derived from gram negative bacteria, in the blood and may result in shock. In equine veterinary practice, the term endotoxemia is often used to describe the severe systemic inflammatory response associated with severe illness, regardless of the underlying etiology. Endotoxemia has been associated with many equine diseases with high mortality. Endotoxemia in equine adults most commonly results from gastrointestinal diseases (Lavoie *et al.* 1990) but also may arise from other gram negative bacterial infections such as peritonitis, pleuritis or metritis (Moore and Barton 2003). In equine neonates, endotoxemia commonly results from gram negative septicemia (Lavoie *et al.* 1990). Approximately 30-40% of horses presented to referral hospitals with clinical signs of colic and 40-50% of neonatal foals presented with septicemia have endotoxin in their systemic circulation (Barton *et al.* 1998b; Steverink *et al.* 1995). Endotoxemia in horses with gastrointestinal diseases is strongly correlated with outcome (Thoefner *et al.* 2001; Tinker *et al.* 1997) and represents a common sequel that increases morbidity and mortality (Hunt *et al.* 1986).

The abnormalities associated with the clinical syndrome of endotoxemia result from a nonspecific innate inflammatory response (McKenzie and Furr 2001). This response has been termed the systemic inflammatory response syndrome (SIRS) (McKenzie and Furr 2001). SIRS, which represents a common terminal phase of the inflammatory response characterized by malignant global activation of multiple pro-inflammatory pathways, is defined by the presence of two or more of the following abnormalities: fever or hypothermia (rectal temperature greater than 39.2°C or less than 37.2°C), tachycardia (heart rate greater than 60 beats per minute), tachypnea (respiratory rate greater than 30 breaths per minute) or hypocapnia (partial pressure of

arterial carbon dioxide less than 32 mmHg), leukocytosis or leukopenia (leukocyte greater than 12,500 or less than 4,000 cells/ul), or increased numbers of immature forms of granulocytes (greater than 10% band neutrophils) (McKenzie and Furr 2001). The changes associated with SIRS can lead to shock, which is characterized by severe hypotension not responsive to intravenous fluid therapy. Shock can result in hypoperfusion and organ dysfunction such that homeostasis cannot be maintained without intervention, a process termed multiorgan dysfunction syndrome (MODS). MODS is a progressive syndrome with initial dysfunction in the cardiovascular system, followed by of other body systems, resulting in the development of refractory hypotension, lactic acidosis, and oliguria, often progressing to death (McKenzie and Furr 2001) (see Figure 2).

While SIRS in horses has traditionally been referred to as endotoxemia, it has become apparent that patients with gram positive bacterial infections, viral infections, trauma, hypovolemia, hemorrhage, and immunologic and drug reactions may exhibit a syndrome of severe systemic inflammation that is clinically identical to that associated with endotoxemia. This has made it clear that the severe inflammatory response observed in all of these conditions is induced by production of pro-inflammatory substances including pro-inflammatory mediators and cytokines (tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6)) (McKenzie and Furr 2001). The inflammatory process itself results solely from the production of endogenous mediators in response to the underlying insult. In summary, endotoxemia is a serious and devastating clinical condition in horses that leads to development of SIRS followed by MODS and eventually death due to an overwhelming systemic inflammatory response.

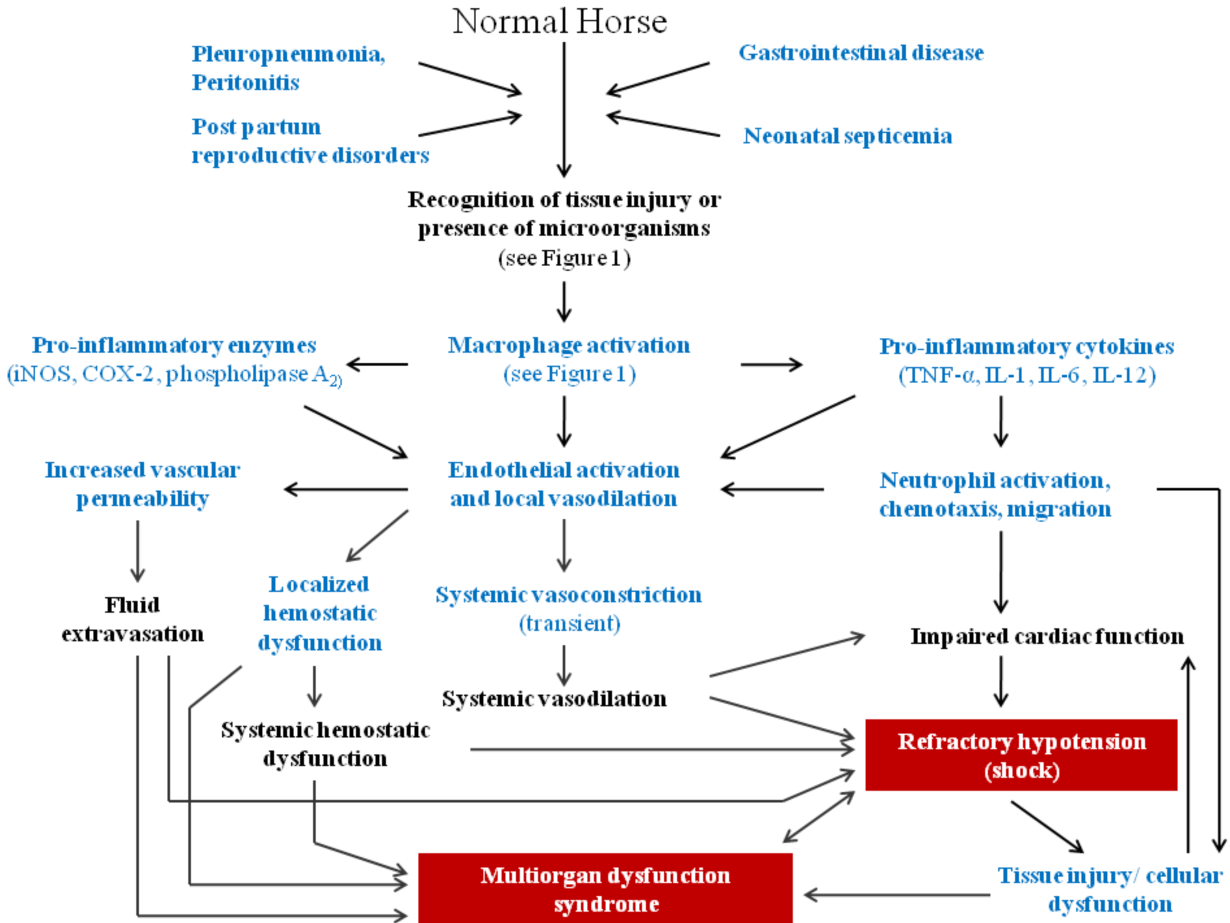

Figure 2. Schematic representation of the progression of the inflammatory process and associated pathophysiologic changes in a horse exposed to gram negative bacterial infections. (Used with permission of H.C. McKenzie III, 2010)

Clinical effects in horses

Numerous studies have been published describing the clinical effects of endotoxin infusion. In general, the signs are similar, but vary with endotoxin dose, duration and route of administration. In horses, clinical signs of endotoxemia consist of tachycardia, abdominal pain, diarrhea, hyperglycemia, thrombocytopenia, systemic hypotension and decreased systemic vascular resistance (Lavoie *et al.* 1990). Endotoxin infusion (0.5 µg/kg bwt) in equine neonates has been shown to lead to depression, anorexia, increased rectal temperature, and initial

leukopenia followed by leukocytosis, hypoglycemia, increased prothrombin time, increased partial thromboplastin time (aPTT), hypertension, increased pulmonary and systemic vascular resistance and mild hypoxemia (Lavoie *et al.* 1990). Marked variations in clinical signs were observed but shock was not induced. In this study, foals appeared healthy once LPS infusion was discontinued. Adult horses given endotoxin (0.1 µg/kg bwt) (*E. coli* O55:B5) exhibited abdominal discomfort, depression, anorexia, pyrexia, tachycardia, tachypnea, altered mucus membrane color, loss of borgorygmi and sweating (Semrad and Moore 1987). Clinical signs varied significantly between individuals. Morris *et al.* infused horses with endotoxin (*E. coli* O55:B5) at a dose of 30 ng/kg bwt in 1L of sterile 0.9% saline over 1 hour with the aim of inducing sublethal endotoxemia (Morris *et al.* 1992). The horses developed lethargy, fever, tachycardia and leukopenia which returned to baseline 6 to 8 hours after the endotoxin infusion. Similar results were observed by Veenman *et al.*, however the duration of effects were more persistent and severe when endotoxin was given at a dose of 2 µg/kg bwt endotoxin (*E. coli* O55:B5) in 60 ml 0.9% saline intravenously over a 2 hour period (Veenman *et al.* 2002). The ponies exhibited fever for 1 to 5 days, inappetence for 24 hours, hyperlactatemia, hemoconcentration, and transient leukopenia.

In a slightly different protocol, Burrows *et al.* administered endotoxin (*E. coli* O26:B6) intravenously (150 µg/kg bwt) once (group 1), intraperitoneally (300 µg/kg bwt) once (group 2), or intraperitoneally (100 µg/kg bwt) four times in 3 hour intervals (group 3) (Burrows 1979). The horses which received intravenous endotoxin exhibited tachycardia, tachypnea, weakness, ataxia and transient recumbency within minutes after the endotoxin administration. Depression and anorexia were apparent for 3 to 4 days. Four horses received a single intraperitoneal dose of endotoxin. The onset of clinical symptoms was slower in this group. Depression, sweating and

cold extremities were observed in all of these horses. Recumbency was noted in three of the four horses that subsequently died. The fourth horse exhibited anorexia and depression for 3 to 4 days before it recovered completely. In the third group, repeated intraperitoneal administration of endotoxin resulted in delayed anorexia and depression of prolonged duration (several days to weeks). Only one horse became recumbent for a short time. Common observations in all three groups included the development of transient fever, hemoconcentration, neutropenia, hyperglycemia, and hyperlactatemia. Mortality varied significantly between the 3 groups (group 1 - 30% mortality, group 2 - 75% mortality, group 3 - 20% mortality) as well as the severity of clinical symptoms (Burrows 1979).

Ward *et al.* demonstrated endotoxemia in horses after intravenous (2, 4, 6 and 6 µg/kg bwt) or intraperitoneal (10, 20, 30 and 30 µg/kg bwt) administration of increasing sublethal LPS doses (*E. coli* O55:B5) in 6 hour intervals (Ward *et al.* 1987). Clinical symptoms included abdominal discomfort, depression, sweating, recumbency, altered mucus membrane color, increased capillary refill time, diarrhea, and absent borgorygmi. Barton *et al.* showed that the intravenous infusion of 20 ng/kg bwt of endotoxin (*E. coli* O55:B5) in 500 ml of 0. 9% sterile saline over a 30-minute period resulted in fever, tachycardia, tachypnea and leukopenia (Barton *et al.* 1998a). All of these studies document that a wide range of low doses of endotoxin (20 ng/kg bwt to 400 µg/kg bwt) induces clinical symptoms in horses. However, depending on the dose and route of administration of LPS, the magnitude and character of the clinical symptoms vary markedly.

These observations are in contrast to studies performed in mice, rats and rabbits. In horses 300 µg of LPS/kg bwt given intraperitoneally is lethal (Burrows 1979), whereas in mice and rats 20 to 25 mg of LPS/kg bwt given intraperitoneally is 100% lethal (Haziot *et al.* 1996;

Sanchez-Cantu *et al.* 1989). A number of studies are listed here to further explain observations in other species. Mice exposed to 10 mg/kg bwt of LPS (*E. coli* o55:B5) intraperitoneally showed lethargy, inappetence and tachypnea (Zhang *et al.* 2009). Another study showed that intraperitoneal injection of 1 mg of LPS in wild type mice resulted in development of shock and only 20% survival within 1 to 5 days after LPS injection (Takeuchi *et al.* 1999). The intravenous administration of 100 µg of LPS in mice resulted in 100% survival, whereas intravenous administration of 200 µg of LPS resulted in only 25% survival within 72 hours after LPS exposure (Berg *et al.* 1995). Gerard *et al.* showed that intravenous administration of 500 µg of LPS resulted in 50% lethality within 72 hours (Gerard *et al.* 1993), contrasting Marchant *et al.* who reported this dose being sublethal and having a 100% survival rate (Marchant *et al.* 1994). Rats were injected intraperitoneally with different doses of LPS and were followed for 72 hours after the LPS challenge (Sanchez-Cantu *et al.* 1989). Naïve rats showed a median lethal dose of 20 to 25 mg/kg of LPS in this study. Rabbits administered 3 doses of LPS (5 µg/kg bwt) (*E. coli* O55:B5) within a 24-hour period developed severe organ injury and/or death within 24 hours of the last LPS administration (Schimke *et al.* 1998). The lethality within this group was 33% and all three LPS injections were required to produce lethal endotoxemia. From these results it is clear that horses are much more sensitive to the effects of LPS in comparison to mice, rats or rabbits.

Mechanisms of endotoxin recognition and activation

Plasma Proteins

Plasma proteins play an important role in mediating responses of cells to bacterial LPS. A number of plasma proteins can bind to LPS and either facilitate or neutralize the effects of LPS on cells. These include:

Lipopolysaccharide-binding protein (LBP). LPS-binding protein (LBP) is an LPS transfer protein that facilitates binding of LPS not only to CD14 but also to lipoproteins (Wurfel *et al.* 1994). LBP is a 58 kDa glycoprotein synthesized in the liver as an acute-phase protein and constitutively secreted into the blood stream (Kirschning *et al.* 1997a). During an acute-inflammatory reaction, serum protein concentrations of LBP rise 5- to 100-fold, depending upon species (Kirschning *et al.* 1997a). LBP binds with high affinity to the lipid A moiety of LPS, thereby forming LPS-LBP complexes which interact with membrane bound CD14 and stimulate macrophages and other CD14-positive cells. LBP can also act as an opsonin to enhance binding of macrophages to LPS or LPS-coated particles and gram negative bacteria (Kirschning *et al.* 1997a; Schumann *et al.* 1990). This binding occurs via a cellular receptor, CD14, which is mobile in the plane of the cell membrane and consecutively triggers an inflammatory response. This function is crucial for controlling infections, and induces monocytic TNF-α production (Jack *et al.* 1997; Schumann *et al.* 1990). LBP enhances the ability of the host to detect LPS early in infection. *In vivo*, LBP is not required for the clearance of LPS but is essential for the rapid induction of an inflammatory response (TNF-α production) by LPS and gram negative bacteria (Jack *et al.* 1997). In addition, LBP restores the ability of LPS-tolerant macrophages to respond to secondary LPS challenge (Mathison *et al.* 1993). Likely mechanisms for the reduced responsiveness of these macrophages to LPS include defective recognition of LPS or deficiency in LPS-specific signal transduction pathways. This phenomenon was only exhibited when the cells where tolerized in the absence of LBP (Mathison *et al.* 1993) which is an unlikely scenario *in vivo*.

LBP in plasma is associated with lipoproteins, suggesting an important role for LBP in the neutralization of LPS by plasma (Wurfel *et al.* 1994). High-density lipoprotein (HDL)

particles alone, reconstituted from highly purified components, were unable to bind or neutralize LPS. Addition of LBP to HDL enabled rapid, potent, dose-dependent binding and neutralization of LPS by HDL. LBP enables neutralization of LPS by facilitating its diffusion from LPS vesicles and micelles into HDL (Wurfel *et al.* 1994). A dominant role for LBP in the neutralization of LPS might be inferred from the observation that LBP is an acute phase reactant, rising from <5 μg/ml to >60 μg/ml after LPS challenge (Calvano *et al.* 1994).

One study in horses determined that LBP concentrations in horses with abdominal pain were higher than in normal horses but LBP concentration did not correlate with outcome or specific disease process (Vandenplas *et al.* 2005). In this study LBP concentration also did not correlate with the presence of systemic endotoxin. Controversial observations regarding the function of LBP in sepsis have been published (Villar *et al.* 2009; Zweigner *et al.* 2001). In low concentrations LBP is believed to enhance the recognition of LPS, thereby activating the innate immune system (Zweigner *et al.* 2001) and having a protective role in severe sepsis. In contrast, high concentrations of LBP in severe sepsis lead to excessive inflammation (Villar *et al.* 2009). LBP is an indicator of severity of lung injury and mortality in humans with severe sepsis (Villar *et al.* 2009). Forty eight hours after the onset of severe sepsis increased LBP concentrations were identified and associated with worse outcome and the highest probability of developing sepsis-induced acute respiratory distress syndrome (ARDS) (Villar *et al.* 2009). Zweigner *et al.* reported contrary findings; high LBP concentrations had an inhibitory role in the LPS-induced inflammatory response (Zweigner *et al.* 2001).

This data leads to the conclusion that moderate concentrations of LBP have a protective role in endotoxemia resulting in activation of the innate immune system. Once extremely high concentrations of LBP are observed, these lead to the induction of excessive inflammation

resulting in SIRS and poor outcome. These effects also depend on the availability of downstream receptors and mediators, therefore an accurate outcome cannot be predicted for each individual. From research conducted in horses, we know that LBP concentrations are increased in endotoxemia but no correlation with outcome was proven. Further research is needed in this field to characterize the role of LBP at different concentrations in horses.

Septin. Septin is a plasma protein complex that binds LPS and mediates its recognition by CD14 receptors. Septin has been identified in human plasma and resembles LBP in several aspects (Wright 1994). In contrast to LBP, it is comprised of multiple protein species which must be simultaneously present to enable its activity. At least two protein species have to be combined to 'activate' septin and to cause LPS effects. LPS is then bound by phagocytes and induces dramatic alterations in the function of leukocyte integrins on polymorph nucleated cells and secretion of TNF-α by monocytes. Septin activity is blocked by addition of protease inhibitors since proteolytic activities are required for opsonization by septin, whereas the function of LBP is not affected (Wright *et al.* 1992). Septin appears to be important for cellular responses to low concentrations of endotoxin that occur in blood during sepsis (LPS < 0.1 ng/ml) (Wright *et al.* 1992). There are no studies that have evaluated the role of septin in horses.

Bactericidal/permeability-increasing protein (BPI). Another soluble protein that binds endotoxin is BPI. BPI belongs to a conserved family of lipid-transfer proteins that includes as its closest relative, the LPS-binding protein (LBP). The primary structures of human LBP and BPI are approximately 45% identical (Kirschning *et al.* 1997b; Schumann *et al.* 1990). BPI has been studied mostly in humans and rabbits; no studies have examined the role of BPI in horses. BPI is mainly expressed in bone marrow in myeloid precursors of neutrophils and stored in primary granules. It can also be detected on the surface of neutrophils and monocytes, presumably

originating from degranulation of neighboring activated neutrophils. BPI is a single-chain cytotoxic cationic protein with a molecular weight of ca. 55 kDa. BPI binds endotoxin close to the lipid A region. The N-terminal domain of BPI is responsible for the endotoxin-neutralizing properties, whereas the C-terminal domain of BPI is needed for BPI-dependent delivery of intact gram negative bacteria and cell-free endotoxin-rich particles to specific host cells (Schultz and Weiss 2007). The binding of BPI to LPS results in the inability of LPS to interact with CD14 receptors. Thus, BPI is a potent LPS neutralizing protein that may limit innate immune responses during gram negative infections (Wittmann *et al.* 2008). BPI has proven to be protective against lethal and sub-lethal challenges with gram negative bacteria and endotoxin (Wittmann *et al.* 2008). Presentation of endotoxin most likely occurs with endotoxin still being an integral component of the outer membrane of gram negative bacteria. The presence of other innate immune-stimulating and antigenic components within the outer membrane makes them primary targets for clearance and resolution of gram negative bacteria/endotoxin-driven inflammation but also potentially important vehicles for delivery of antigenic material to APCs (Schultz and Weiss 2007). BPI is found in plasma in much lower concentrations than LBP. LBP and BPI compete for LPS binding and this competition determines the response of the organism to LPS.

High-density lipoproteins (HDL). Plasma lipoproteins, particularly high-density lipoproteins (HDL), bind and neutralize LPS (Munford *et al.* 1981; Rudbach and Johnson 1964; Ulevitch *et al.* 1979); by acting synergistically with LBP (Wurfel *et al.* 1994). HDL particles alone, reconstituted from highly purified components, were unable to bind or neutralize purified LPS. Addition of LBP to HDL, however enabled rapid, potent, dose-dependent binding and neutralization of LPS by HDL (Wurfel *et al.* 1994). LBP enables neutralization of LPS by facilitating its diffusion from LPS vesicles and micelles into HDL (Wurfel *et al.* 1994).

Disaggregation of LPS precedes the binding of LPS to HDL and results in lipoprotein-bound LPS which is less toxic. *In vivo* studies have revealed that rats naturally have high concentrations of HDL and are therefore more resistant to LPS than other species (Munford *et al.* 1981).

Soluble CD14 receptor (sCD14). CD14 is present in a soluble form (sCD14) in blood or as a glycosylphosphatidylinositol (GPI)-linked form (mCD14) on the cell surface of monocytes, macrophages and neutrophils (Werners *et al.* 2005). Binding of LPS to soluble CD14 (sCD14) prevents LPS binding to membrane-bound CD14 (mCD14) thereby inhibiting cellular activation. However, sCD14-LPS complexes can activate cells which do not themselves express mCD14, however extremely high concentrations of LPS (ten times the lethal dose) are required to activate endothelial cells via sCD14-LPS complexes (Haziot *et al.* 1996). This is unlikely to have biological significance *in vivo*. In addition sCD14 may act as a shuttle molecule like LBP to transfer LPS to HDL, thus neutralizing its toxic effects (Schutt 1999). It appears that sCD14 most importantly is involved in neutralization of LPS *in vivo*.

Plasma protease inhibitor. Tissue factor pathway inhibitor (TFPI) is a Kunitz type plasma protease inhibitor that inhibits factor Xa and the factor VIIa/tissue factor catalytic complex, and thereby results in feedback inhibition of the coagulation cascade. Kunitz type plasma protease inhibitors are proteins that function as protease inhibitors and are specific for either trypsin or chymotrypsin. Proteins from the Kunitz family contain from 170 to 200 amino acid residues and one or two intra-chain disulfide bonds. TFPI exists in three different pools (1) associated with lipoproteins, (2) stored in platelets, and (3) associated with endothelium such that it can be released into circulation by heparin. TFPI binds to endotoxin *in vitro* and prevents the interaction of endotoxin with LBP and CD14, thereby blocking the cellular responses (Park *et al.*

1997). But it is important to point out that once sCD14-LPS complexes were formed, TFPI could not block the cellular responses, therefore it is only effective pre-LPS-exposure. Natural TFPI concentrations may not be sufficient for the attenuation of the response to endotoxin but the administration of TFPI may be considered as a treatment option (Park *et al.* 1997). Animals treated with TFPI in experimental settings showed a significantly decreased IL-6 response (Creasey *et al.* 1993). It has also been shown that TFPI concentrations are significantly increased in septic patients leading to the attenuation of responses to endotoxin (Carson *et al.* 1991; Sandset *et al.* 1989; Takahashi *et al.* 1995; Warr *et al.* 1989).

The listed plasma proteins have not been investigated in detail in the horse. Their neutralizing effects of LPS seem to be efficacious when exposed to minor amounts of LPS but inadequate when exposed to overwhelming gram negative bacterial infections leading to systemic endotoxemia and endotoxic shock. LBP plays a major role in binding LPS, forming LPS-LBP complexes and activating the downstream inflammatory cascade.

Membrane-associated molecules

Membrane-bound CD14 (mCD14). Cluster of differentiation antigen 14 (CD 14) is a 55 kDa glycoprotein that is attached to the cell membrane of monocytes, macrophages, and neutrophils via a phosphatidylinositol (PI) glycan anchor. The PI glycan anchor is essential for the presence of CD14 on the cell surface (Wright *et al.* 1990). CD14 is responsible for the recognition of LBP and binding of LPS-LBP complexes (Wright *et al.* 1990). Once LPS-LBP complexes are bound to CD14, LPS is integrated into the phospholipid layer of the cell membrane. LPS and gram negative bacteria can be internalized by this LBP-dependent pathway. CD14 is necessary for the blood monocytes to respond to low concentrations of LPS (0.01-1

ng/ml) in order to induce an innate immune response. At LPS concentrations of > 10 ng/ml *in vitro* the synthesis of TNF-α is induced without the presence of LBP or CD14.

LPS, TNF-α, and IFN-ɣ upregulate CD14 expression on human myeloid cells *in vitro* (Schutt 1999). Inhibition of the CD14 pathway represents an experimental method to prevent septic shock (Leturcq *et al.* 1996) in primates and rabbits (Schimke *et al.* 1998). The administration of CD14-antibodies was effective in preventing septic shock even when administered after LPS exposure. In horses infused with endotoxin, the expression of CD14 on PBMCs increases and a correlation between this enhanced expression and the clinical signs of endotoxic shock was reported (Kiku *et al.* 2003). CD14 is involved in the clearance of gram negative pathogens during infection and improves the sensitivity of the immune system to infection (Haziot *et al.* 1994).

Toll-like receptors (TLRs). Toll-like receptor 4 (TLR4) is the principal LPS-signal-transduction molecule (Hirschfeld *et al.* 2000). TLRs are essential pattern recognition receptors in cells of the innate immune system. TLRs consist of an extracellular domain containing multiple leucine-rich repeats (LRRs), a transmembrane domain and an intracellular Toll/interleukin-1 receptor domain (TIR) (Rock *et al.* 1998). In mammalian species, there are at least 11 cloned TLRs. Toll-like receptor 4 (TLR4) was first characterized in man and is expressed predominantly in cells from the innate immune system (Fujihara *et al.* 2003). After ligand binding, TLR4 dimerizes and undergoes conformational changes necessary for the recruitment of downstream signaling molecules (Akira and Takeda 2004). LPS-associated proteins (LAPs) are cell-surface proteins, such as heat shock protein 70 (Hsp70), Hsp90 and chemokine receptor 4 (CXCR4), which are distinct from CD14 and TLRs and can bind LPS both dependently and independently of mCD14 (Triantafilou *et al.* 2001). Downstream signal

transduction through TLRs is dependent on myeloid differentiation primary response gene 88 (MyD88), a cytoplasmic adapter protein. MyD88 is associated with the serine-threonine protein kinase interleukin-1 receptor-associated kinase (IRAK). IRAK is then phosphorylated and associated with the tumor necrosis factor-associated factor 6 (TRAF-6) adapter protein, which then activates two pathways, one being the mitogen-activated protein kinase (MAPK) pathway and the other being the nuclear factor-kappa B (NF-κB) pathway (Chaplin 2010; Medzhitov and Janeway 2000) (see Figure 3).

Myeloid differentiation protein 2 (MD2). LPS signaling through TLR4 also depends on the presence of MD2 on the cell surface (Gruber *et al.* 2004; Nagai *et al.* 2002). TLR4 and MD2 are processed through the trans- Golgi-apparatus secretory pathway and reside on the cell surface as a mature protein complex (Latz *et al.* 2002; Werners *et al.* 2005). After binding of LPS to LBP and CD14, LPS is recognized by MD2 which binds the lipid A part of LPS (Werners *et al.* 2005). MD2 then associates with the extracellular domain of TLR4 thereby activating intracellular phosphorylation cascades including the MAPK and NF-κB activation pathways leading to the production of pro-inflammatory cytokines (Werners *et al.* 2005). Both pathways (MAPK and NF-κB) result in increased DNA transcription and production of various inflammatory mediators, including cytokines such as TNF-α, IL-6, and IL-1 (Werners *et al.* 2005).

<u>Intracellular Activation Pathways</u>

Nuclear factor-kappa B (NF-κB) pathway. Nuclear factor-kappa B (NF-κB) is a nuclear transcription factor that plays a significant role in the induction of pro-inflammatory mediators.

In its inactivated state, it is normally bound to inhibitor κB (I κB). As a result of this binding, it remains localized to the cytosolic compartment. Activation of NF-κB is mediated by a serine kinase known as inhibitor κB kinase (IKK). IKK itself is activated by the binding of lipopolysaccharide (LPS) to the Toll-like receptor 4 (TLR4) or via the effects of TNF-α or IL-1. Once activated, serine phosphorylation of I κB results in its destruction and the subsequent nuclear translocation of NF-κB (Marik and Raghavan 2004) which initiates the transcription of a number of genes that are involved in the inflammatory response (See Figure 3). NF-κB family is composed of various members, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel, which can form homo- and heterodimers (Ryseck *et al.* 1992; Schmitz and Baeuerle 1991). Numerous studies have shown that the transactivator form of NF-κB is the p65 subunit whereas the p50/p50 homodimer has a minimal transactivation capacity (Fan and Cook 2004; Franzoso *et al.* 1992). Upon LPS stimulation, p50/p65 heterodimer complex is predominant, which leads to gene transactivation (Fan and Cook 2004) and transcription of cytokine mRNA.

Mitogen-activated protein kinase (MAPK, MAP3K) pathway. Five distinct groups of MAPKs have been characterized, of which the extracellular regulated signaling kinase 1 and 2 (ERK), c-Jun amino-terminale kinases (JNK) and p38 are the most extensively studied (Werners *et al.* 2005) (see Figure 3). The MAPKs are regulated by phosphorylation cascades upon LPS stimulation and each different group of MAPKs has different effects. The p38 proteins regulate environmental stresses and release of inflammatory cytokines (Werners *et al.* 2005). The transcription factor activator protein 1 (AP-1) is activated by the MAPKs and translocates into the nucleus where it upregulates gene expression and controls cellular processes including differentiation, proliferation and apoptosis.

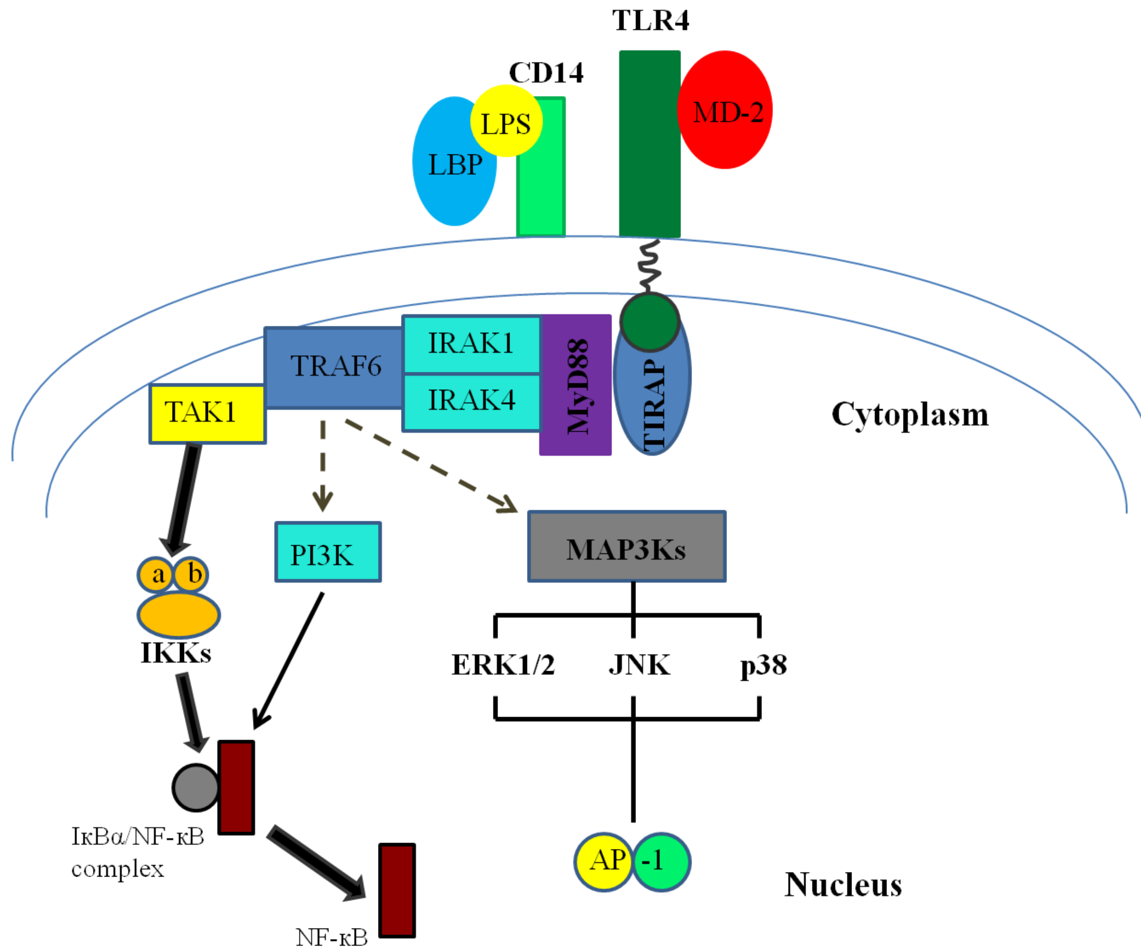

Figure 3. Activation of signal transduction pathways after binding of LPS-LBP complex to membranous CD14. A number of protein kinases are involved in the signal transduction from the toll-like receptor to the final translocation of NF-κB and AP-1 into the nucleus followed by production of inflammatory mediators and cytokines. (TIRAP-Toll-interleukin 1 receptor domain containing adaptor protein; IRAK1-Interleukin-1 receptor-associated kinase 1; IRAK4-Interleukin-1 receptor-associated kinase 4; TRAF6-TNF receptor-associated factor 6; TAK1-TGF-beta activated kinase 1; PI3K-phosphatidylinositol 3-kinases; AP-1-activator protein 1)

LPS-induced Cellular Production of Inflammatory Mediators

A wide variety of cytokines and inflammatory mediators are induced by cellular exposure to endotoxin and cellular activation via the preceding mechanisms. Three of the most commonly studied cytokines, TNF-α, IL-1 and IL-6, have common effects in the defense against pathogens.

They induce pyrexia and promote the antibacterial activity of leukocytes. IL-6 induces the production of acute phase proteins in the liver. Their ultimate function is to aid removal of the pathogens by the body's phagocytic cells (Werners *et al.* 2005). However, overexpression may result in deleterious effects resulting from the derangement of the normal function of the cytokine network leading to septic shock, multiple organ failure and death.

Inflammatory mediators

TNF-α. TNF-α is the earliest mediator of the LPS response (MacKay *et al.* 1991). Its production is induced within 15-30 minutes after LPS administration and usually peaks at 90-120 minutes after LPS exposure. TNF-α possesses cytotoxic activity and is a potent inducer of fever and the production of several other pro-inflammatory cytokines.

TNF-α is synthesized as a 26 kDa type II transmembrane precursor that is displayed on the plasma membrane. The TNF-α precursor is proteolytically cleaved to yield a biologically active 17 kDa mature TNF-α that forms a trimer in solution. During the interaction of effector and target cells, membrane-bound pro- TNF-α in the effector cells binds TNF-α receptors on the target cells, and induces TNF-α responses via receptor aggregation (Kriegler *et al.* 1988). Pro-TNF-α is a more potent activator of TNF-α receptor II than mature TNF-α (Grell *et al.* 1994). TNF-α is produced by numerous immune cells (monocytes/macrophages, NK cells, Kupffer cells, B cells, and T cells) in response to an assortment of activating stimuli. The most important stimuli are LPS, T-cell receptor activation, crosslinking of surface immunoglobulin, viral infections, parasites, and the presence of IL-1 and TNF-α.

The expression of TNF-α is tightly controlled, because systemic overproduction of TNF-α activates inflammatory responses, and mediates hypotension, diffuse coagulation, and widespread tissue damage (Wang *et al.* 2003). TNF-α is a pleiotropic pro-inflammatory cytokine that exerts multiple biologic effects. TNF-α induces fever and anorexia via hypothalamic centers. Cardiovascular effects include the increased permeability of vascular endothelial cells resulting in capillary leakage syndrome, enhanced tissue factor expression and suppression of protein C resulting in endotoxic shock which may lead to multiple organ failure and death. TNF-α expression also has been found to induce insulin resistance, gastrointestinal ischemia, colitis, hepatic necrosis and decreased albumin production.

TNF-α is the most important and most proximal mediator of the severe systemic inflammatory response and the administration of TNF-α antibodies prevents the development of septic shock (Barton *et al.* 1998a). High TNF-α and/or IL-6 concentrations have been associated with an unfavorable outcome (Barton and Collatos 1999; MacKay *et al.* 1991; Steverink *et al.* 1995). IL-10 blocks the *in vitro* and *in vivo* production of TNF-α and decreases LPS toxicity as well as LPS-induced mortality in humans and mice (Gerard *et al.* 1993). Upregulation of TNF-α, IL-1 and IL-6 and their detrimental effects have been described in horses with endotoxemia (MacKay and Socher 1992; Morris *et al.* 1992; Veenman *et al.* 2002). MacKay *et al*. evaluated TNF-α as a marker of cytotoxicity after endotoxin infusion. One anesthetized adult horse was given a possibly lethal dose of 100 μg of LPS/kg bwt (*E. coli* O111:B4) in order to quantify TNF-α production in response to LPS. The horse was euthanized 2 hours after the LPS infusion. TNF-α was first detected 30 minutes after the LPS infusion and was still increasing (4,910 U/ml) at the time of euthanasia. They also exposed 3 foals to a high dose of 5 μg of LPS/kg bwt intravenously 30 minutes after administration of flunixin meglumine (1.1 mg/kg bwt) which is

known to minimize the clinical effects of LPS without reducing the TNF-α response. TNF-α was first detected 30 minutes after the LPS infusion, peaked within 2 hours at 2,668 ±797 U/ml), and then decreased rapidly to baseline. These foals exhibited fever, tachycardia and tachypnea within 2 to 4 hours after the LPS infusion. Four adult horses received 30 ng of LPS/kg bwt/h intravenously for 4 hours. This infusion resulted in mild clinical signs of transient fever and leukopenia. TNF-α was detected within 1 hour of LPS infusion, peaked within 2 hours at 15-20 U/ml, and decreased to baseline rapidly.

Recently, several *in vivo* and *in vitro* studies have examined the expression of pro-inflammatory cytokines in response to LPS in horses. Neuder *et al.* reported an increased expression of TNF-α, IL-1, IL-6 and IL-8 in equine PBMCs after exposure to 10 ng/ml LPS *in vitro* (Neuder *et al.* 2009). Sun *et al.* exposed equine monocytes to 100 pg/ml LPS *in vitro* and reported an increased expression of TNF-α, IL-1, IL-8, IL-10 and COX-2 (Sun *et al.* 2010). Nieto *et al.* described the pro-inflammatory cytokine profile in horses after infusion of 30 ng/kg LPS (Nieto *et al.* 2009). The expression of IL-1α, IL-1β, IL-6, IL-8, and TNF-α were increased after LPS administration and peaked at 60 minutes post-infusion except for IL-6 which peaked at 90 minutes post-LPS. This also confirms that IL-6 expression is induced by other cytokines that are synthesized early after LPS exposure.

IL-1. IL-1 is a pro-inflammatory cytokine that is synthesized as precursor molecule without a signal peptide. After removal of N-terminal amino acids by specific proteases, the resulting peptides are called 'mature' forms. The 31 kDa precursor form of IL-1β is biologically inactive and requires cleavage by a specific intracellular cysteine protease. The mature form of IL-1β is a 17.5 kDa molecule. Other proteases can also process the IL-1β precursor extracellularly into an active cytokine (Coeshott *et al.* 1999). Membrane-bound IL-1α is

biologically active and is found on the surface of monocytes and B lymphocytes. IL-1 initiates the COX-2 pathway, type 2 phospholipase A and inducible NO synthase (iNOS) resulting in the production of large amounts of PGE2, PAF and NO. IL-1 also promotes the infiltration of inflammatory and immunocompetent cells into the extravascular space (Dinarello 2003).

IL-1-deficient mice are characterized by absence of acute phase response, anorexia, pyrexia, and are incapable of IL-6 expression but still respond to LPS exposure (Zheng *et al.* 1995). Therefore we can conclude that IL-1 is a strong downstream mediator in endotoxemia but LPS effects are not IL-1 dependent.

IL-6. Interleukin-6 is a 21-28 kDa glycoprotein cytokine produced by various cells including fibroblasts, endothelial cells, T and B lymphocytes, mesangial cells, and keratinocytes; however, the main sources of IL-6 are monocytes and macrophages (Robinson *et al.* 1993). IL-6 plays a key role in host defense, regulating antigen-specific immune responses, hematopoiesis, cellular differentiation, and the acute phase reaction subsequent to inflammatory insult (Robinson *et al.* 1993). The concentration of IL-6 in compartmentalized body fluids and in the circulation is high in animals with infectious, traumatic, autoimmune, and neoplastic diseases (Hirano 1992; Van Snick 1990). Gram negative bacterial endotoxin is a potent stimulus for IL-6 production, acting either directly (Fong *et al.* 1989) or indirectly through induction of other cytokines, such as IL-1 and TNF-α (Fong *et al.* 1989; Shalaby *et al.* 1989).

Serum IL-6 concentration is high during endotoxemia, and measurement of IL-6 concentration has been evaluated as a prognostic indicator in clinical cases of bacterial sepsis and endotoxemic shock (Hack *et al.* 1989; Morris *et al.* 1992). In endotoxemic shock, IL-6 is significantly elevated at the onset and rapidly decreases as endotoxic shock progresses. The

expression of IL-6 is associated with the concentration of TNF-α and correlated with the clinical outcome (Calandra *et al.* 1991; Yoshimoto *et al.* 1992). In horses, IL-6 concentrations have predictive value for unfavorable outcome and the simultaneous presence of increased LPS and TNF-α (Steverink *et al.* 1995). Increased concentrations of IL-6 have been associated with a poor clinical condition and outcome in horses (Steverink *et al.* 1995).

The role of IL-6 in endotoxemia has not been fully defined. Potentially beneficial effects of IL-6 production during endotoxemia have been described and include decreased production of TNF-α and IL-6 by monocytes, and inducing production of the full range of acute-phase proteins, including protease inhibitors that are postulated to limit tissue damage (Robinson *et al.* 1993). Other studies, however, have indicated that IL-6 may contribute to the pathogenic effects of gram negative infections or intravenous administration of TNF-α and that administration of IL-6 antagonists may be beneficial in endotoxemia (Leon *et al.* 1992; Starnes *et al.* 1990). LPS infusion in neonatal foals led to an increased production of IL-6 (Robinson *et al.* 1993). A group of colostrum fed foals developed higher concentrations faster than colostrum deprived foals; therefore high IL-6 concentrations are thought to be immunostimulatory and may protect foals from septicemia (Robinson *et al.* 1993). These findings lead to the conclusion that IL-6 concentrations of less than 50 ng/l have immunostimulatory effects but IL-6 concentrations of equal or greater than 50 ng/l are associated with overwhelming inflammation and poor outcome.

IL-12. IL-12 is an important regulator of the inflammatory response to endotoxin and is essential for clearance of intracellular toxins and infections. IL-12 is mainly produced by antigen-presenting cells (APCs), including monocytes, macrophages and dendritic cells (DCs) (Kalinski *et al.* 2003). IL-12 production is induced by pathogen-related products signaling via CD14, TLR-2, TLR-9, CD11b/CD18, and by phagocytosis of bacteria (Kalinski *et al.* 2003).

Pathogen-related agents capable of inducing IL-12 production include bacterial LPS, whole bacteria, nucleic acids, RNA, lipoteichoic acid, heat shock proteins and microparticulate ingestion (Kalinski *et al.* 2003; Sutterwala *et al.* 1997). The second type of IL-12 inducing stimulus is the interaction of APCs with T helper (Th) cells (Kalinski *et al.* 2003). Biologically active IL-12 is composed of two subunits, p35 and p40 (Kalinski *et al.* 2003). IL-12 secretion by macrophages can be up- or downregulated by other cytokines. IFN-ɣ and IL-1β enhance IL-12 production through co-stimulatory effects (Kalinski *et al.* 2003). IL-4, IL-10, IL-13, glucocorticoids, PGE_2, histamine, TGF-β, and IFN-α inhibit the production of IL-12 (Kalinski *et al.* 2003; Sutterwala *et al.* 1997). IL-12 production is strongly enhanced in IL-10 deficient mice (Kalinski *et al.* 2003).

IL-12 enhances cell-mediated (type-1) immunity, Th1-type responses to $CD4^+$ T cells and functions of B cells, APCs, vascular and stromal cells (Kalinski *et al.* 2003). IL-12 is the primary and by far the best studied Th1 inducing factor (in mice and humans) (O'Garra and Arai 2000). The lack of IL-12 results in an inability to develop Th1 responses and to fight intracellular infections (Kalinski *et al.* 2003; Stobie *et al.* 2000). IL-12 enhances the production of IL-10 by B cells (Skok *et al.* 1999) and T helper cells (Assenmacher *et al.* 1998) which represents a negative feedback mechanism for prevention of IL-12 mediated damage during chronic inflammation. IL-12 enhances cytolytic effector function via proliferation of NK and T cells (Kalinski *et al.* 2003). IL-12 also prevents apoptosis of T cells, NK cells, APCs and DCs (Kalinski *et al.* 2003). IL-12 promotes B cell production of IL-10, initiating a B cell-dependent shift from Th1 to Th2 responses (Skok *et al.* 1999). IL-12 promotes nuclear localization of NF-κB and may prime murine DCs for IL-12 production (Grohmann *et al.* 1998). IL-12 acts as a macrophage

chemoattractant (Kalinski *et al.* 2003). The expression of IL-12 has not been investigated in horses and therefore not been associated with equine endotoxemia.

IL-10. IL-10 is an 18-21 kDa polypeptide produced by activated T cells, B cells, monocytes/macrophages, mast cells and keratinocytes in response to many pathogens (bacterial wall components, parasites, fungi, viral components) (Ding *et al.* 2003). IL-12 can induce IL-10 mRNA expression and protein synthesis in NK cells. In severe infections and stress conditions, cytokines, hormones and arachidonic acid derivates are released and upregulate IL-10 synthesis in monocytes, macrophages and T cells (Ding *et al.* 2003; Marchant *et al.* 1994). Elevated IL-10 expression is associated with septicemia in humans (Marchant 1994).

IL-10 is a key regulator of immune responses (Ding *et al.* 2003). IL-10 inhibits monocyte and macrophage synthesis of IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α and reactive oxygen and nitrogen intermediates (de Waal Malefyt *et al.* 1991). IL-10 is involved in antigen presentation to Th1 cells, chemokine expression by monocytes and the bactericidal response of macrophages to IFN-ɣ. IL-10 inhibits NK cell production of IFN-ɣ and T cell-dependent responses of B cells (Ding *et al.* 2003). In sum, it suppresses multiple immune responses through actions on T cells, B cells, and APCs and shifts the immune response from Th1 to Th2 (Ding *et al.* 2003). The shift to a Th2 response counteracts the pro-inflammatory effects of the Th1 response and establishes a well-balanced Th1 and Th2 response that is suited to the immune challenge which is especially important for the development of endotoxin tolerance.

IL-10 has been shown to effectively modulate the cytokine syndrome caused by endotoxin by inhibiting the production of pro-inflammatory cytokines (Ding *et al.* 2003). IL-10 has protective effects in experimental endotoxemia, and exogenous administration rescues mice

from LPS-induced toxic shock, which is correlated with reduced levels of serum TNF-α (Gerard *et al.* 1993; Howard *et al.* 1993). IL-10 inhibits production of TNF-α, regulates hemodynamic parameters, microvascular permeability and reduces mortality in experimental murine endotoxemia (Hickey *et al.* 1998; Standiford *et al.* 1995). Mice treated with anti-IL-10 from birth or IL-10 deficient mice are more susceptible to endotoxin induced shock then normal mice (Berg *et al.* 1995). Limited data is available regarding the expression of IL-10 in equine PBMCs after *in vitro* LPS exposure (Sun *et al.* 2010; Sykes *et al.* 2005).

TGF-β. Transforming growth factor-β (TGF-β) is synthesized as a 390-amino-acid glycosylated pre-protein that contains a 29-amino-acid hydrophobic signal sequence which is cleaved, resulting in pro-TGF-β1 (Derynck *et al.* 1985). After further cleavage and homodimerization the biologically active 25 kDa dimeric polypeptide of TGF-β is derived. TGF-β is a potent inducer of apoptosis but also exhibits anti-apoptotic and pro-survival actions. TGF-β inhibits ceramide-induced apoptosis, induces liver atrophy and apoptotic cell death in the liver. NF-κB promotes cell survival and anti-apoptotic effects on hepatocytes and lymphocytes. TGF-β increases I κB (via CD40) which is correlated with NF-κB downregulation and apoptosis, therefore TGF-β has a critical role in promoting cell survival. Limited data is available in the literature concerning TGF-β expression in human and murine models of endotoxemia (de Waal Malefyt *et al.* 1991; Karp *et al.* 1998; Randow *et al.* 1995). In the horse, the expression of TGF-β has not been investigated in models of endotoxemia.

Treatment of endotoxic shock in horses

No therapeutic agents to date are efficacious in protecting patients from endotoxin-mediated tissue damage and organ failure (Russell 2006). Supportive care including intravenous fluid therapy, anti-inflammatory drugs, broad spectrum antimicrobials, polymyxin B, hyperimmune plasma and DMSO are commonly employed (Sykes and Furr 2005; Werners *et al.* 2005).

Nonsteroidal anti-inflammatory drugs (NSAIDs). Nonsteroidal anti-inflammatory drugs are commonly used in the treatment of endotoxemia, of which the most important is probably flunixin meglumine. Low doses of flunixin (0.25 mg/kg bwt q8hr) inhibited eicosanoid production and suppressed blood lactate elevation without masking all of the physical manifestations of endotoxemia necessary for accurate clinical evaluation of the horse's status (Semrad *et al.* 1987). Flunixin meglumine is effective if given pre-endotoxin exposure but has little impact once responses are already initiated (Semrad and Moore 1987). Pretreatment with 1.1 mg/kg bwt of flunixin meglumine is more effective than low dose flunixin meglumine treatment at ameliorating tachycardia, tachypnea and fever induced by LPS (Moore *et al.* 1981) and has been shown to increase time till death in fatal models of endotoxemia in the horse (Ewert *et al.* 1985; Templeton *et al.* 1987). Flunixin meglumine at 1.1 mg/kg bwt maintained cardiovascular function and peripheral perfusion during experimental endotoxemia in ponies (Ward *et al.* 1987).

Pentoxifylline. Pentoxifylline inhibits TNF-α, IL-6 and tissue factor activity in a dose-dependent manner following *in vitro* exposure of equine whole blood to LPS (Barton and Moore

1994) with the most significant effects occurring when pentoxifylline was administered one hour prior to LPS exposure. Reproduction of these effects *in vivo* in the horse have only shown minimal positive effects on clinical signs (prevention of pyrexia and tachypnea) but no effect on TNF-α or IL-6 expression (Barton *et al.* 1997). In this study, pentoxifylline was administered intravenously immediately after endotoxin infusion. Therefore, we can conclude that pentoxifylline has only limited beneficial effects when administered after the endotoxin exposure.

Glucocorticoids. Glucocorticoids (GCs) are physiological inhibitors of inflammatory reactions and are used in the treatment of many inflammatory disorders. Glucocorticoids bind to the cytosolic glucocorticoid receptor (GP). Once this receptor is activated it translocates into the nucleus where it interacts with specific transcription factors (AP-1 and NF-κB) and prevents the transcription of pro-inflammatory genes. GCs inhibit the production of inflammatory cytokines, including TNF-α and IL-10, by LPS activated monocytes/macrophages and protect animals from LPS-induced lethality (Beutler *et al.* 1986; Fantuzzi *et al.* 1994; Gonzalez *et al.* 1993; Marchant *et al.* 1996). Endogenous GCs are produced during the course of inflammatory responses, including endotoxic shock (Marchant *et al.* 1996). Inhibition of the biosynthesis or effects of endogenously produced GCs in mice increases LPS-induced TNF-α production and lethality (Fantuzzi *et al.* 1995; Gonzalez *et al.* 1993; Marchant *et al.* 1996). Marchant *et al.* showed that methylprednisolone inhibited TNF-α production in a murine model of endotoxemia (Marchant *et al.* 1996). Low dose methylprednisolone (2 and 20 mg/kg) had no effect on IL-10 expression but high doses of methylprednisolone (50 mg/kg) significantly increased serum levels of IL-10 in this model (Marchant *et al.* 1996). Weichhart *et al*. stated that the route of corticosteroid administration is important in determining its efficacy in treatment of endotoxic shock

(Weichhart *et al.*). Intraperitoneal administration of dexamethasone failed to prevent endotoxin-induced death (Weichhart *et al.*), whereas subcutaneous administration of dexamethasone completely inhibited LPS-induced lethality in mice (Weichhart *et al.*). Intravenous administration of methylprednisolone is used in equine neonates to treat SIRS, however use of corticosteroids in adults is usually discouraged due to the risk of laminitis.

Polymyxin B. Polymyxin B is a basic cationic cyclic polypeptide antimicrobial with a broad range of activity against gram negative bacteria that also neutralizes LPS (Morresey and Mackay 2006). Polymyxin B functions as a chelating agent, binding the lipid A subunit of LPS in a ratio of 1:1, thereby neutralizing it (Morresey and Mackay 2006). Interaction of LPS with humoral and cellular receptors is prevented, and initiation of a pro-inflammatory cascade is avoided. Polymyxin B has dose-dependent effects. At high doses (equal to or greater than 15,000 units/kg bwt) the drug is a bactericidal antimicrobial with predominantly gram negative spectrum of activity. At these high doses polymyxin B has neurotoxic and nephrotoxic side effects in horses. At lower doses (1,000 to 5,000 units/kg bwt) the drug binds the lipid A component of LPS and safely and effectively reduces or eliminates LPS-induced events (Barton *et al.* 2004). Treatment with polymyxin B prior to and after LPS exposure reduced fever, tachycardia and serum TNF-α concentration in horses (Barton *et al.* 2004; Parviainen *et al.* 2001).

New therapeutics. While promising new therapeutics are currently being studied, they have not been evaluated in clinical patients. A synthetic lipid A analogue E5564 (Figueiredo *et al.* 2008), a phospholipid emulsion (Moore *et al.* 2007) and a selective MAPK inhibitor (specific p38 MAPK inhibitor) (Neuder *et al.* 2009) have been investigated in equine PBMCs *in vitro* and inhibited the expression of pro-inflammatory cytokines.

Figueiredo *et al.* reported the effectiveness of synthetic lipid A analogue E5564 treatment in horses. The hydrophobic lipid A region of LPS is responsible for initiating various innate immune responses that result in development of the SIRS. Because the lipid A region is conserved among a variety of gram negative bacteria, this molecule is an attractive target for the development of LPS antagonists (Figueiredo *et al.* 2008). The compound E5531 has been successfully used as an LPS antagonist in murine and human cells (Figueiredo *et al.* 2008) in experimentally induced endotoxemia. E5531 induces strong pro-inflammatory responses in equine cells and can therefore not be used in horses (Figueiredo *et al.* 2008). E5564 is a second generation synthetic lipid A analogue. It is more potent, has longer duration of action, and is more stable than E5531 (Figueiredo *et al.* 2008). E5564 blocks the action of LPS at its cell-surface receptor (TLR4) and prevents the induction of cellular mediators in rodents and humans (Figueiredo *et al.* 2008). The efficacy of E5564 was evaluated in an *in vitro* experiment. E5564 inhibited the LPS-induced production of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10) in a concentration-dependent manner (Figueiredo *et al.* 2008). Therefore, E5564 appeared to be the first lipid A analogue that has potential as an effective therapeutic agent in horses with endotoxemia. Further studies are needed to support its clinical use in equine patients (Figueiredo *et al.* 2008). The synthetic lipid A analogue E5564 is currently being evaluated in phase III clinical trials in humans (Figueiredo *et al.* 2008).

Moore *et al.* investigated the administration of a phospholipid emulsion as a possible new therapeutic in endotoxemia. As described earlier, HDLs neutralize LPS (Wurfel *et al.* 1997) and human patients with low serum concentrations of lipoproteins have an increased risk for infection and reduced prognosis for survival (Gordon *et al.* 1996). Administration of phospholipid emulsions in endotoxemia has resulted in improved survival (Goldfarb *et al.* 2003)

and significant attenuation of LPS effects in humans (Gordon *et al.* 2005) and horses (Winchell *et al.* 2002). Winchell *et al.* reported gross hemolysis in horses given the phospholipid emulsion (200 mg/kg) intravenously (Winchell *et al.* 2002). Moore *et al.* administered a lower dose of the phospholipid emulsion (100 mg/kg) reducing hemolysis, yet still achieving attenuation of LPS effects. The horses received 30 ng/kg of LPS (*E. coli* O55:B5) immediately after the phospholipid emulsion was given. Improved clinical scores, prevention of pyrexia and tachycardia, and reduced production of TNF-α were reported in treated horses (Moore *et al.* 2007). Further *in vivo* studies are required to evaluate its effectiveness in clinical settings.

Neuder *et al.* investigated a specific p38 MAPK pathway inhibitor for treatment of endotoxemia in horses. The p38 MAPK pathway is a member of MAPK signal transduction pathway in the cellular downstream cascade of induction of pro-inflammatory cytokine production after LPS exposure. SB203580 and SB202190 are specific p38 MAPK inhibitors and decrease protein and mRNA expression of COX-2 in LPS-stimulated equine leukocytes (Neuder *et al.* 2009). In an *in vitro* experiment, equine PBMCs were treated with SB203580 or SB202190 and then exposed to LPS (10 ng/ml) (*E. coli* O55:B5) for 2 or 4 hours. SB203580 significantly decreased IL-1β and IL-8 mRNA expression. SB202190 significantly decreased TNF-α and IL-6 mRNA expression. The p38 MAPK inhibitors exhibited differences in specificity and potency, and further studies are needed to evaluate these as potential therapeutics in equine endotoxemia.

Research. Given that the toxicity of endotoxin is due to its ability to produce widespread over-stimulation of immune response cells, one approach which is currently being investigated in humans and laboratory animals is immunologic manipulation to attenuate the host response to endotoxin. Following the observation that a low dose of endotoxin can mitigate future host

responses to endotoxin, further studies to develop 'endotoxin tolerance' as a therapeutic modality are underway.

Endotoxin Tolerance (ET)

Endotoxin Tolerance (ET) is defined as a reduced capacity of the host (*in vivo*) or of cultured macrophages/monocytes (*in vitro*) to respond to LPS activation following a first exposure to this stimulus (Fan and Cook 2004). ET was first studied by Beeson in a rabbit model (Beeson 1947). ET is not a whole scale down-regulation of signaling protein and inflammatory mediators, since LPS tolerant animals and cells still respond to LPS challenge by expressing specific genes and proteins. Instead, ET appears to be a specific adaptive response that is mediated by a complex, regulated process (Fan and Cook 2004; West and Heagy 2002). The result of ET is a profound reduction in the clinical signs associated with endotoxin exposure, a phenomenon that has obvious potential clinical applications.

ET is characterized by inhibition of LPS-responsive inflammatory pathways including: TNF-α, IL-8 and IL-12 production, IL-1 and IL-6 release, COX-2 activation, MAPK activation and NF-κB translocation (Werners *et al.* 2005). The inhibition of TNF-α production has become a useful phenotypic marker of endotoxin tolerance due to its central role as a mediator of LPS-induced inflammation, and its down-regulation during ET.

Duration of ET

Endotoxin tolerance occurs in two stages: early phase endotoxin tolerance (EPET) and late phase endotoxin tolerance (LPET) (Allen *et al.* 1996; Greisman *et al.* 1969). EPET, which is a transient phenomenon that occurs within hours to days and does not involve production of antibodies to endotoxin, has been demonstrated in the horse as well as in humans and rabbits

{Allen, 1996 #361;(Greisman *et al.* 1969). Altered host responses after the second dose of endotoxin included a decreased duration of fever, attenuated TNF-α response and decreased mortality (Allen *et al.* 1996; Greisman *et al.* 1969; Rayhane *et al.* 1999). The early phase of ET is associated with a down-regulation of LPS-induced inflammatory mediator production. EPET occurs within 48 hours of endotoxin exposure and then rapidly wanes. The late phase of ET is mediated by anti-endotoxin antibodies against the 'O' domain and common core antigens, which blunt the release of endogenous pyrogen from macrophages (Greisman *et al.* 1969). LPET was described in rabbits, humans and rats (Greisman *et al.* 1969; Mulholland *et al.* 1965; Sanchez-Cantu *et al.* 1989). LPET develops after the initial 48 hours after endotoxin exposure and reaches its maximum effectiveness at 8 to 10 days post endotoxin exposure. Recovery of responsiveness to LPS was observed at day 20 post endotoxin exposure in rats (Sanchez-Cantu *et al.* 1989). In rabbits ET lasted for up to 34 days (Mulholland *et al.* 1965). In human volunteers duration of ET for as long as 17 weeks was described (Neva and Morgan 1950). In these studies, humans and animals were exposed to a wide dose range (0.25µg to 250 µg of LPS in rats and rabbits; 0.001 to 0.3 µg/kg bwt in humans; 0.5 to 50 µg/kg bwt in rabbits) of different kinds of endotoxin (*E. coli* O127:B8; *Salmonella enteritidis*; *Salmonella typhimurium* endotoxin; *Salmonella typhosa* endotoxin; *Pseudomonas* endotoxin) (Greisman *et al.* 1969; Mulholland *et al.* 1965; Sanchez-Cantu *et al.* 1989). Thus, the duration of ET depends on the dose of endotoxin administered as well as the kind of endotoxin given. It was also shown that in LPET 'cross-tolerance' can be induced between bacterial endotoxins from different gram negative bacterial species (Greisman *et al.* 1969).

The mechanisms for the induction and maintenance of endotoxin tolerance are not completely understood, but recent investigations have also demonstrated a critical role for the cytokines IL-12, IL-10 and TGF-β (Shnyra *et al.* 1998). Tolerization is accomplished without significant effect upon resistance to infections. Resistance to gram negative infections, phagocytosis, and fungal infections, for example, are actually enhanced in tolerized laboratory rodents (West and Heagy 2002).

<u>In vivo *ET*</u>

ET has been induced in many animal models and *in vitro* studies. Rats that received a pretreatment exposure to endotoxin *in vivo* survived a subsequent challenge of endotoxin that was 100% lethal for naïve rats (Sanchez-Cantu *et al.* 1989). This was associated with markedly decreased concentrations of circulating TNF-α in pretreated rats. In this study, duration of ET was also assessed. Maximal refractory effects to LPS were reached on days 3 to 5 after the induction of ET (Sanchez-Cantu *et al.* 1989). A slow recovery of the responsiveness to LPS followed this period; on day 20 full responsiveness to LPS was re-established (Sanchez-Cantu *et al.* 1989). ET was induced in mice with the following protocol: priming dose of 25 μg of LPS given in the footpad followed by 200 μg of LPS intravenously 24 hours later. This protocol resulted in development of ET and decreased TNF-α production.

The phenomenon of endotoxin tolerance has also been demonstrated in the horse. Pretreatment with a single dose of 50 ng/kg bwt of O55:B5 *E. coli* LPS was associated with an attenuated clinical response and decreased endogenous production of TNF-α in horses given a subsequent LPS challenge (50 ng/kg bwt of LPS) (Allen *et al.* 1996). Fevers and changes in blood pressure and respiratory rate were all blunted in horses on secondary exposure. In addition, the endogenous TNF-α response was almost completely eliminated in horses after the second,

challenge, infusion of LPS. Burrows *et al.* injected 4 doses of 100 μg/kg bwt of LPS (*E. coli* O26:B6) intraperitoneally at 3 hour intervals into adult ponies (Burrows 1979). These ponies (group 3) were compared to two groups of ponies who received a single dose of LPS either intravenously (group1) or intraperitoneally (group 2). Depression and anorexia were observed in all 3 groups but were least marked in group 3. Fever was most significant in group 3 but lethality was markedly decreased in group 3 (20% lethality) compared to the other 2 groups and suggested a reduced LPS response after repeated LPS exposure (Burrows 1979). It is clear; therefore that endotoxin tolerance can be induced in the horse, yet the mechanisms of this phenomenon have not been systematically investigated in the horse.

<u>In vitro *ET*</u>

ET was induced in murine peritoneal macrophages by incubation with a tolerizing dose of 10 ng/ml of LPS for 20 hours, followed by incubation with a challenge dose of 10 ng/ml of LPS for up to 2 hours (Medvedev *et al.* 2000). In tolerized murine peritoneal macrophages the MAPK pathway, degradation of I κBα and I κBβ, and activation of the transcription factors NF-κB and AP-1 were inhibited, suggesting that ET results from impaired function of common LPS signaling intermediates (Medvedev *et al.* 2000). In a different model, murine peritoneal macrophages were pretreated with a range of LPS (*E. coli* O111:B4) concentrations of 0.1 to 10 ng/ml for 6 hours, and then stimulated with 1 μg/ml of LPS for 24 hours. A marked reduction in TNF-α production was observed (Shnyra *et al.* 1998). ET resulted in down-regulation of IL-12 and up-regulation of IL-10. The peak of Il-12 production coincided with the peak in TNF-α production; significant correlation between the levels of IL-12 and TNF-α was also observed (Shnyra *et al.* 1998). ET was demonstrated in porcine CD14 positive monocytes *in vitro* after pretreatment with either 0.1, 1 or 10 μg/ml of LPS (*Salmonella typhimurium* L 2262) for 20

hours followed by exposure to 10 µg/ml of LPS for 4 and 24 hours. Development of ET was associated with reduced production of TNF-α and IL-8. ET was induced in several *in vitro* experiments with human cells. Human monocytes were cultured with 100 ng/ml of LPS (*Salmonella enteritidis*) for 24 hours followed by a second exposure to LPS (10 µg/ml) for 24 hours (Peck *et al.* 2004). In tolerized cells, production of TNF-α was decreased to less than 1% of non-tolerized cells (Peck *et al.* 2004). IL-6 production was increased in tolerized cells compared to non-tolerized cells. However, no change was observed in IL-1β and IL-8 production (Peck *et al.* 2004). In another study, human dendritic cells were pretreated with 1 ng/ml LPS (*Salmonella typhimurium*) for 24 hours, and then stimulated with 1 µg/ml of LPS (Karp *et al.* 1998). ET was successfully induced, TNF-α and IL-12 production were markedly reduced.

Mechanisms for induction and maintenance of ET

Several mechanisms and pathways are involved in the development of ET. Experimental evidence suggests that alterations in signal transduction after repeated exposure to endotoxin are critical to development of ET (West *et al.* 1996; West *et al.* 1997; Ziegler-Heitbrock *et al.* 1994) (see Figure 4).

LBP and CD14. High concentrations of LBP and sCD14 have been associated with neutralization of endotoxin in septic human patients (Kitchens *et al.* 2001). Increased expression of mCD14 (Ziegler-Heitbrock *et al.* 1994) and sCD14 (Labeta *et al.* 1993) is associated with the development of ET and has been described in animals and humans. Other models reported no change in mCD14 expression in humans, rabbits and mice (Mathison *et al.* 1993; McCall *et al.* 1993; Ziegler-Heitbrock *et al.* 1997). In 2003, Heagy *et al.* reported that mCD14 is not involved in the development of ET in human monocytes (Heagy *et al.* 2003).

TLR and downstream signal transduction. Upregulation of TLR4 after LPS exposure has been described (Jiang *et al.* 2000) leading to NF-κB activation. Decreased phosphorylation of the MAP kinase pathway (Durando *et al.* 1998; Medvedev *et al.* 2001; Ropert *et al.* 2001; West *et al.* 2000), and IRAK pathway (Li *et al.* 2000) lead to impaired pro-inflammatory cytokine expression. Sato *et al.* described down regulation of the cell surface (TLR)4-MD2-complex in ET which resulted in decreased inflammatory cytokine production, blocked activation of IRAK or NF-κB, and altered TLR4-MyD88 dependent signaling (Sato *et al.* 2000). Inhibition of TLR4 expression was also reported in macrophages from mice and hamsters (Medvedev *et al.* 2001; Nomura *et al.* 2000).

Nuclear factor κB (NF-κB) pathway. Inhibition of the NF-κB pathway has been described in ET and is caused by an increased level of the inactive p50 homodimer relative to the active p50/p65- heterodimer. ET leads to a suppressed degradation of I κBα and I κBβ and impaired LPS-stimulated activation of the transcription factor of NF-κB, (Kastenbauer and Ziegler-Heitbrock 1999; Medvedev *et al.* 2000; Wahlstrom *et al.* 1999; West *et al.* 2000; Ziegler-Heitbrock 1995) and an increased expression of I κBα or impaired degradation of I κBα (Blackwell *et al.* 1997; Kohler and Joly 1997; Medvedev *et al.* 2001; Shames *et al.* 1998; Wahlstrom *et al.* 1999).

Ligation of the macrophage Fcɣ receptor. From research in laboratory rodents, it appears that tolerance to endotoxin can be manipulated and induced by means other than endotoxin exposure. For example, macrophages can be tolerized to endotoxin by *ex vivo* ligation of the macrophage Fcɣ receptor with IgG-opsonized erythrocytes (Gerber and Mosser 2001). In addition, adoptive transfer of the tolerized macrophages induced resistance to a lethal endotoxin dose in recipients. This was associated with upregulation of IL-10 and down regulation of IL-12

(Gerber and Mosser 2001; Sutterwala *et al.* 1997). These may prove to be clinically applicable methods of inducing endotoxin tolerance in horses, but the fundamental phenomenon needs further investigation in the horse.

IL-1. Mice pretreated with IL-1 prior to LPS exposure developed ET. In this study, TNF-α expression remained elevated; therefore, a different mechanism for ET induction was suspected than in ET associated with low TNF-α expression (Leon *et al.* 1992).

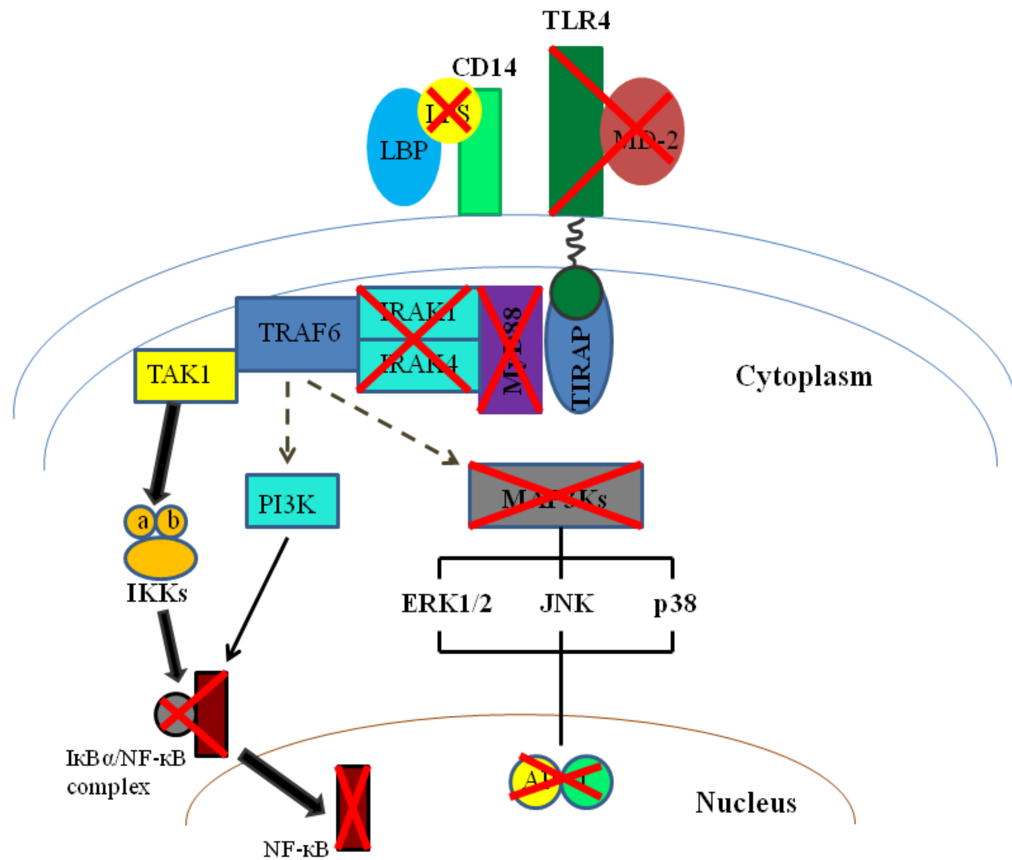

Figure 4. Blockage/inactivation of numerous signal transduction pathways result in impaired translocation of NF-κB and AP-1 and development of endotoxin tolerance (ET). LPS may be neutralized prior to interacting with LBP and CD14.

IL-12. It has been demonstrated in numerous studies in humans and laboratory animals that exposure to endotoxin results in the release of the pro-inflammatory cytokine IL-12 (Shnyra *et al.* 1998). Pretreatment (priming) of human PBMCs with LPS blocked IL-12 production (Karp *et al.* 1998). In murine peritoneal macrophages that were primed IL-12 expression was down-regulated (Shnyra *et al.* 1998), this down-regulation is associated with improved survival (Heinzel *et al.* 1994; Karp *et al.* 1998; Wysocka *et al.* 1995; Zisman *et al.* 1997). In humans it has been shown that IL-12 suppression associated with ET may result in impaired bacterial

clearance and an inability to respond appropriately to secondary infections in survivors of sepsis (Karp *et al.* 1998). IL-12 suppression in ET is caused by a different mechanism than TNF-α suppression and is not dependent on IL-10 or TGF-β in mice or humans (Karp *et al.* 1998; Sutterwala *et al.* 1997). The response of IL-12 to single or repeated endotoxin administration in the horse is not reported.

IL-10. IL-10 is an anti-inflammatory cytokine which regulates the response to endotoxin and can prevent endotoxic shock. In IL-10 deficient mice the lethal dose of endotoxin was 20 times lower than in normal mice (Berg *et al.* 1995). When ET was induced in IL-10 deficient mice the tolerizing dose was 100 times lower than in normal mice. Despite these findings, IL-10 does not appear to be a main effector of ET (Berg *et al.* 1995) but a critical component of the host's natural defense against pathological responses to LPS. IL-10 downregulates the production of pro-inflammatory cytokines, mainly TNF-α, and reduces LPS toxicity and mortality in ET in mice and humans (de Waal Malefyt *et al.* 1991; Flohe *et al.* 1999; Gerard *et al.* 1993; Howard *et al.* 1993; Marchant *et al.* 1994). IL-10 mediates protection in the earliest phase of the LPS response but ET does not appear to be IL-10 dependent. IL-10 was produced within 18-24 hours *in vitro*; but *in vivo* IL-10 peaks at the same time as TNF-α (Beutler *et al.* 1986).

IL-10 is required for development of ET in human monocytes (de Waal Malefyt *et al.* 1991; Gerard *et al.* 1993; Howard *et al.* 1993; Randow *et al.* 1995) *in vitro*. In murine cells controversial findings have been reported. In a model of ET in murine peritoneal macrophages IL-10 was up-regulated (Shnyra *et al.* 1998). Administration of IL-10 has been shown to reproduce some aspects of ET but IL-10 knockout mice could still be tolerized in one study (Berg *et al.* 1995; Cavaillon and Adib-Conquy 2006).

In one study of *ex vivo* endotoxin stimulated equine PBMCs, IL-10 was found to peak 6 hours after endotoxin exposure, coincident with TNF-α peak, then to persist for up to 24 hours, while TNF-α mRNA concentration decreased (Sykes *et al.* 2005). No data regarding IL-10 expression in ET in equine cells exist.

TGF-β. TGF-β functions in close relationship with IL-10. Only few studies have investigated its importance in endotoxemia and ET. Recently, rabbit macrophages (Mathison *et al.* 1990) or human monocytes and macrophage cell lines (Ziegler-Heitbrock *et al.* 1992) have been shown to acquire a status of ET by preculture with low doses of LPS *in vitro*. The anti-inflammatory mediators IL-10 and TGF-β seem to be of critical importance in this *in vitro* tolerance (Flohe *et al.* 1999). Antibodies against IL-10 and TGF-β interfered with the development of an *in vitro* tolerance in human monocytes (Randow *et al.* 1995) but did not prevent the suppression of IL-12 expression. Therefore, IL-10 and TGF-β have essential roles in the development of ET in humans (Karp *et al.* 1998; Randow *et al.* 1995). The production of TGF-β has not been investigated in the horse following endotoxin exposure or in ET.

Although the mechanisms of induction and maintenance of ET are not completely understood, from these studies it is clear that at least one mechanism for the maintenance of endotoxin tolerance is the reciprocal modulation of IL-10 and IL-12 responses. Further research in this field is needed to characterize this relationship more specifically.

Conclusion

The current literature offers substantial information regarding endotoxemia and ET in different species. From published studies, we know that substantial differences exist in the immune response to LPS across species. Therefore, species-specific research is clearly needed. Equine endotoxemia has been examined closely and is well characterized; but little is known about the production of IL-12 and anti-inflammatory cytokines in equine endotoxemia. ET has been induced in horses but poorly investigated. No information regarding the expression of IL-12 and anti-inflammatory cytokines such as IL-10 and TGF-β exists. The goals of this study were to establish an *in vitro* model of ET in equine PBMCs and to characterize the expression of IL-12 and IL-10 in tolerized equine PBMCs.

Chapter 2. Induction of Endotoxin Tolerance *in vitro* in equine PBMCs

The pathophysiologic derangements associated with the presence of circulating LPS in horses cause significant morbidity and mortality in the horse (Allen *et al.* 1996). While gastrointestinal diseases remain the leading cause of death in horses (Tinker *et al.* 1997) mortality within this group is related to the degree of endotoxemia (Thoefner *et al.* 2001). It has been reported that 30% of horses examined for colic at referral institutions, and 50% of septicemic foals, have detectable concentrations of endotoxin in their blood at the time of admission (Barton 2003). While gastrointestinal diseases are common clinical conditions in which endotoxemia arises, endotoxemia also results from a number of other clinical settings, including grain overload, gram negative bacterial pleuropneumonia, peritonitis and uterine infections.

In the horse, as in other species, most of the clinical signs associated with endotoxemia are due to the presence of circulating cytokines (Moore 1998). Although endotoxin stimulates an array of cytokine and inflammatory mediators, only a few key cytokines have been described in equine endotoxemia. Tumor necrosis factor-α (TNF-α), IL-1 and IL-6 are key pro-inflammatory cytokines released during endotoxemia in horses (Moore 1998; Steverink *et al.* 1995; Veenman *et al.* 2002). These cytokines are important in the onset and development of septic shock and a direct relationship between blood concentrations of TNF-α, IL-6 and mortality has been established in horses, humans and rodents (Steverink *et al.* 1995; Veenman *et al.* 2002). TNF-α is important in the pathogenesis of sepsis, as indicated by its increased concentration during experimental and natural sepsis and by the fact that administration of exogenous TNF-α mimics the clinical and pathological changes of sepsis (Moore and Morris 1992; Veenman *et al.* 2002). The importance of TNF-α in horses with endotoxemia is demonstrated by the observation that

horses with gastrointestinal disease exhibit an increased concentration of TNF-α in serum and peritoneal fluid (Barton *et al.* 1996; Steverink *et al.* 1995).

In species other than the horse it has been demonstrated that anti-inflammatory cytokines are also stimulated following endotoxin exposure, including IL-4, IL-10, IL-11, IL-13, TGF-β, soluble TNF-α receptors and IL-1 receptor antagonist (Krishnagopalan *et al.* 2002; Roy 2004). The expression of pro-and anti-inflammatory cytokines other than TNF-α, IL-1 and IL-6 are poorly reported in the equine literature. IL-10 mRNA has been shown to be up-regulated in horses after exposure to endotoxin (Sykes and Furr 2005; van den Hoven *et al.* 2006) . It is likely that this cytokine plays a key role in down-regulation of the profound inflammatory response to endotoxin.

A phenomenon called 'endotoxin tolerance' (ET) represents a reduced capacity of animals or cells to respond to LPS activation following a first exposure to this stimulus. ET is associated with improved clinical signs and decreased mortality. ET has been induced in horses *in vivo* but the cytokine profile associated with ET in horses has not been investigated.

The blunted response to endotoxin in ET has potential clinical utility in manipulating the cellular response to endotoxin in equine patients. Endotoxin tolerance represents a novel approach to the treatment of endotoxemia in the horse. The experiment described below is intended to demonstrate that endotoxin tolerance can be induced in horses *in vitro*, and to describe the cytokine responses which are associated with its development, and necessary for its maintenance. Once this mechanism is established, protocols can be devised and tested to induce this phenomenon in horses experiencing clinical endotoxemia. The establishment of a

mechanism and testing platform (*in vitro* tolerization), as described in this report, is a critical first step in that process.

The purpose of the study was to describe a method for inducing endotoxin tolerance in equine PBMCs *in vitro*, and to investigate part of the cytokine profile associated with endotoxin tolerance, specifically, TNF-α, IL-12 and IL-10. Tolerance was defined as a reduction in TNF-α production by greater than 75% when compared to non-tolerized positive control cells. Once the tolerizing protocol was determined, the experiment was repeated and the cytokine profile of tolerized PBMCs was examined by quantitative PCR. The hypothesis was that mRNA expression of IL-12 and IL-10 from tolerized cells differs from that of positive control cells.

Materials and Methods

Horses

Six healthy mature horses were used. Horses ranged from 5 to 20 years of age. There were two mares and four geldings in the group. The horses were maintained in one group on pasture turnout with free choice access to water at all times. None of the horses received any medication during the study period and none of them had previously been exposed to endotoxin for experimental studies. The study was conducted at the Marion duPont Scott Equine Medical Center.

All horses were determined to be healthy by physical examination, complete blood cell count and serum biochemistry prior to inclusion in the study. The protocol was approved by the Virginia Tech Institutional Animal Care and Use Committee.

Blood collection, PBMC recovery and cell culture

Sixty ml of blood were collected aseptically from the left jugular vein of the horses into lithium heparinized vacutainers[a] and gently mixed. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described (Sykes *et al.* 2005). Briefly, the heparinized blood was centrifuged at 600 x *g* for 10 minutes at 20°C. The buffy coat was aspirated and suspended in 8 ml of RPMI 1640 incomplete medium[b]. Next 2 to 3 ml of this suspension was gently layered onto 4 ml of ficoll-hypaque (Lymphoprep®[c]) (density 1.077 g/ml) in 15 ml conical, sterile screw top vials[d] and centrifuged at 350 x *g* for 30 minutes at 20°C. This method was repeated for the entire sample. The mononuclear cells were recovered from the Lymphoprep®-cell interface by gentle aspiration, resuspended in incomplete RPMI 1640 medium and centrifuged at 600 x *g* 5 minutes. The cells were washed in incomplete RPMI 1640 medium three times, then resuspended in RPMI 1640 complete medium consisting of RPMI 1640 incomplete medium[b], 10% heat-inactivated fetal bovine serum (FBS)[e], penicillin[f] (50 IU/ml), streptomycin[f] (50 IU/ml), HEPES buffer (25 mmol) and L-glutamine[g]. Cell counts were determined using a hemocytometer[h]. Cell viability was assessed with trypan blue staining[i]. 0.5 ml of the cell suspension was mixed with 0.1 ml of 0.4% Trypan blue stain. This was allowed to stand for 5 minutes at room temperature (15-25°C). A hemocytometer was used to count viable cells. Absolute cell counts were determined and cell suspensions were diluted in complete RPMI 1640 medium to a concentration of 1×10^6 viable cells/300 µl, which was placed into each well of a multiwell culture plate for subsequent determination of TNF-α production at 12 hours.

The experiment comprised three experimental groups: a tolerized group, a negative control group and a positive control group. In this study, negative control cells are defined as cells that had not been exposed to endotoxin at any time point. Positive control cells were cells that had not been tolerized but did receive the ‘challenge’ dose of endotoxin. Tolerized cells were those cells that

received a low ‘tolerizing’ dose of endotoxin subsequently followed by a second ‘challenge’ dose of endotoxin.

Prior to tolerization, cells were incubated with either 0.1 ng/ml, 1 ng/ml or 10 ng/ml of endotoxin. The doses of LPS (Lipopolysaccharides from *E. coli* O55:B5)[k] were selected based on previously published work (Barton *et al.* 1996; Shnyra *et al.* 1998; Sykes *et al.* 2005). LPS was dissolved in incomplete RPMI 1640 medium at a final concentration of 1 mg/ml. Complete RPMI 1640 medium was added to all wells to achieve a total volume of 1 ml. The plates were incubated at 37°C with 5% carbon dioxide (CO_2) for 6 hours then centrifuged at 3500 RPM for 3 minutes after which the supernatant was aspirated. The cells were washed 3 times with 0.9% NaCl then resuspended in complete RPMI 1640 medium. Positive control and tolerized cells then received a ‘challenge’ dose of either 1 ng/ml, 10 ng/ml or 100 ng/ml of endotoxin. The plates were incubated at 37°C with 5% CO_2 for 6 hours, after which supernatant from each well was removed, placed in polypropylene tubes and frozen at -70°C until assayed. Treatments were replicated 3 times.

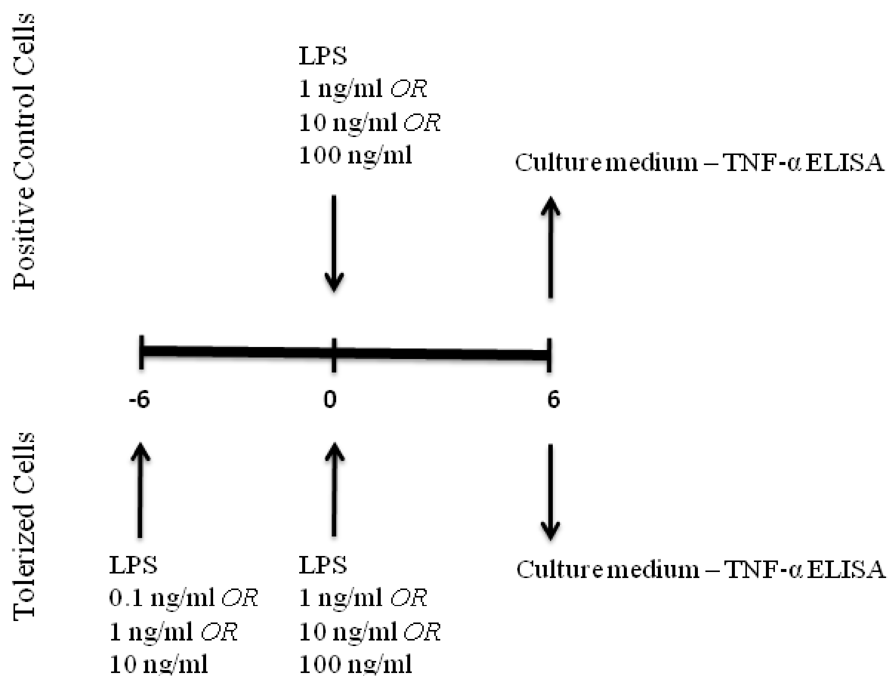

Figure 5. Experimental design of phase I. Negative control cells were not exposed to LPS (not shown). Positive control cells were exposed to 'challenge' doses of LPS only. Tolerized cells received 2 doses of endotoxin ('tolerizing' and 'challenge' dose) 6 hours apart.

TNF-α assay

Cell culture supernatants were assayed for TNF-α concentration with a commercially available equine TNF-α assay[l]. The assay was performed as previously described (Sun *et al.* 2008). ELISA plates were prepared as described. Briefly, TNF-α antibodies were reconstituted in carbonate/bicarbonate buffer (1:100 dilution) and 100 µl were added to each well of a 96-well plate and incubated overnight at room temperature (22-25°C). The following day, the plates were washed 3 times, and incubated for 1 hour with 300 µl of blocking buffer with 4% bovine serum albumin[e] in Dulbecco's PBS[m]. After washing 3 times, 100 µl of 1:100 detection antibody was added and the plates incubated for 1 hour at room temperature, after which they were washed

three times with wash buffer (300 µl per well). 100 µl of Streptavidin-Horseradish Peroxidase (SA-HRP) (1:400) was added to each well then incubated for 30 minutes at room temperature. After washing, 100 µl of TMB Substrate solution was added to each well and the plates were incubated in the dark for 20 minutes at room temperature. After the addition of 100 µl of Stop Solution (Sulfuric Acid) the absorbance was measured at 405nm using a micoplate reader[o]. Cells were considered to be tolerized if the TNF-α concentration was reduced by more than 75% of the positive control sample for the same horse.

Statistical analysis

Data were entered into a desktop computer, and after data entry validation, analysis was performed with a computerized statistical package: SAS[p]. Differences of TNF-α expression between treatment groups and treatment time were evaluated by use of mixed-model repeated measures ANOVA. Pair-wise comparisons were made on significant differences identified with ANOVA using Tukey's post hoc test. TNF-α concentrations were reported as means ± SD. Results were considered significant at a value of $P < 0.05$.

Results

TNF-α was produced by all cells exposed to endotoxin (positive control cells and tolerized cells). The magnitude of TNF-α production showed a wide variability between individual horses and between the dosages of endotoxin.

ET in tolerized cells was defined as reduction of TNF-α production by greater than 75% compared to positive control cells. Reduction in TNF-α production ranged from 75% to 100%, and was observed in all cells treated with all 3 tolerizing doses. A statistically significant difference in TNF-α concentration ($P < 0.0001$) was identified between positive control cells and

tolerized cells. There was no statistical difference present between the different 'tolerizing' and 'challenge' doses that were used to induce ET. From these results, we chose a 'tolerizing' dose of 1 ng/ml of LPS and a 'challenge' dose of 10 ng/ml of LPS for the second phase of the study.

TNF-α concentration

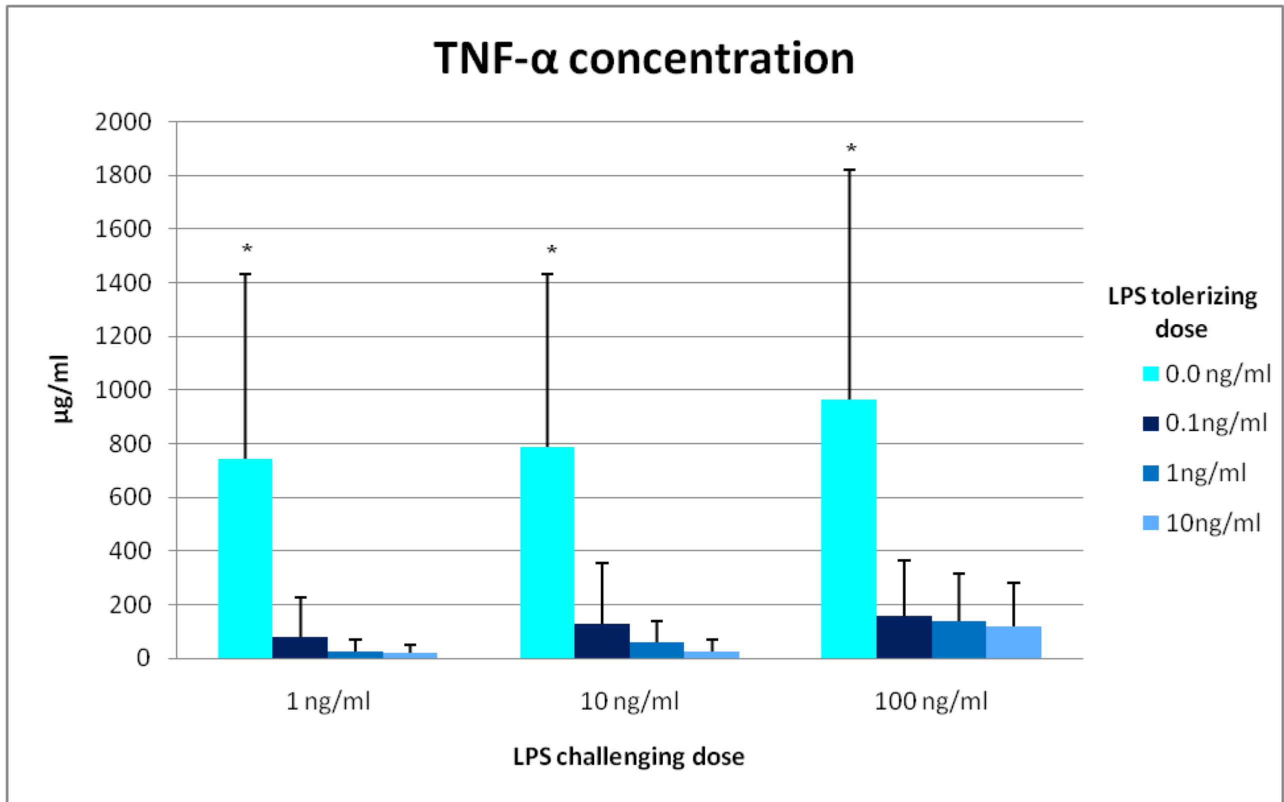

Figure 6. TNF-α concentration for positive control cells and tolerized cells at different doses of endotoxin exposure. Data presented as mean ± SD. Asterisk (*) denotes statistically significant difference ($P < 0.0001$) between positive control cells and tolerized cells.

Conclusion

An *in vitro* model for induction of ET was successfully developed. The ‘tolerizing’ dose of 1 ng/ml of endotoxin followed by a ‘challenge dose’ of 10 ng/ml of endotoxin was associated with ET most reliably. This protocol can be recommended for *in vitro* induction of ET for further evaluation and characterization of ET.

Chapter 3. Characterization of Endotoxin Tolerance *in vitro* in PBMCs

Materials and Methods

Horses

Six healthy mature Thoroughbred mares were used for the study. Horses ranged from 10 to 19 years of age. The horses were maintained in one group on pasture turnout with free choice access to water at all times at the Middleburg Agricultural Research and Extension (MARE) center. None of the horses received any medication during the study period and none of them had been exposed to endotoxin previously for experimental studies. The study was conducted at the Middleburg Agricultural Research and Extension (MARE) center and the Marion duPont Scott Equine Medical Center.

All horses were determined to be healthy by physical examination, complete blood cell count and serum biochemistry prior to inclusion in the study. The protocol for the project was approved by the Virginia Tech Institutional Animal Care and Use Committee.

Blood collection

Seven hundred ml of blood were collected aseptically into heparinized[q] blood collection bags[r] from the left jugular veins of the Thoroughbred mares. The blood was gently mixed in the collection bags and immediately transported to the Marion duPont Scott Equine Medical Center. Blood was transferred into 50 ml sterile conical tubes for centrifugation and PBMCs were isolated as described in Chapter 2. Absolute cell counts were determined as described above and cells were suspended in complete RPMI 1640 medium at a concentration of 1 x 10^6 cells/200 µl.

Cell culture and sample collection

Based on the results from Phase I, a 'tolerizing' dose of 1 ng/ml of endotoxin and a 'challenge' dose of 10 ng/ml of endotoxin were selected for induction and testing of endotoxin tolerance, respectively.

The three treatment groups included: a tolerized group, a positive control group and a negative control group: all samples were cultured in triplicates. Three million cells were put into each well of a 48 well culture plate and the total volume was adjusted to 1 ml by the addition of complete RPMI 1640 media. Cells were tolerized by incubation with 1 ng/ml of LPS and incubated at 37°C with 5% CO_2 for 6 hours. Following this, the plates were centrifuged at 3500 RPM for 3 minutes and the supernatant and cell pellets were recovered, protease inhibitor[s] was added (1:100) to the supernate and it was stored at -70°C until assayed. The cell pellets were used immediately for RNA isolation and cDNA synthesis. The cells to be used for challenge testing were resuspended in incomplete RPMI 1640 and were washed as described above. Then the cells were resuspended in complete RPMI 1640 medium and the challenge dose of 10 ng/ml of endotoxin was added to the positive control and tolerized samples. The total volume in each well was 1 ml. Plates were incubated at 37°C with 5% CO_2. After 6, 12 and 24 hours the cell culture supernatants and cell pellets were recovered as described above. Protease inhibitor was added to the cell culture supernatants according to the manufacturer's instructions, the samples were frozen immediately at -70°C until assayed. The cell pellets were used immediately for RNA isolation and cDNA synthesis.

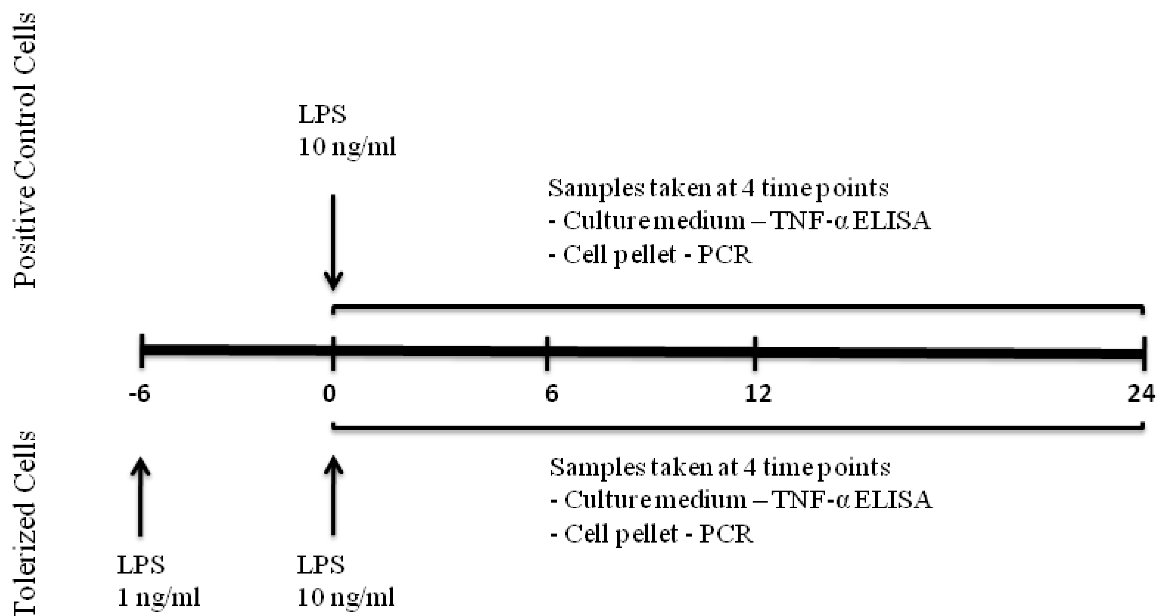

Figure 7. Experimental design of phase II. Negative control cells were not exposed to LPS (not shown). Positive control cells were exposed to 'challenge' dose of LPS only. Tolerized cells received 2 doses of endotoxin ('tolerizing' and 'challenge' dose) 6 hours apart. Culture medium and cell pellets were collected at 4 time points from all cell groups.

RNA isolation, cDNA synthesis and gene expression

The RNA isolation was performed with a commercially available kit[t] using the technique previously described by Sykes *et al.* (Sykes *et al.* 2005). Briefly, cell pellets were disrupted and homogenized in guanidium lysis buffer, precipitated with ethanol and purified by use of a commercially available column based protocol. This protocol included an on-column DNase[u] digestion and a subsequent RNA cleanup procedure to exclude genomic template contamination.

RNA concentration was measured by optical density at 260 nm (spectrophotometer). RNA quality was assessed by gel electrophoresis on a denaturing agarose gel[v]. One microgram of

RNA in each sample was converted to cDNA with a commercial transcription kit[w] and oligo (dT) primers using the manufacturer's protocol. Briefly, 1 µg of total RNA was mixed with Reverse Transcription Master Mix containing Reverse Transcription buffer, Deoxynucleotide Triphosphate mix, Reverse Transcription random primers, Multiscribe™ Reverse Transcriptase and nuclease-free water. Mixtures of each sample were placed in each well of a multiwell plate and cDNA was synthesized in triplicates. The mixtures were incubated at 25°C for 10 minutes, then 37°C for 2 hours and heated at 85°C for 5 minutes. The samples were stored at -70°C until used for PCR analysis.

The expression of IL-10 and IL-12 was quantified by real-time PCR[y]. Triplicate samples recovered from each experimental group at time 0, 6, 12 and 24 were used and target cDNAs were amplified via real-time PCR by use of *Taq* DNA polymerase (TaqMan®)[x] and equine gene specific primers designed from available published sequences (Garton *et al.* 2002) (Appendix A). Real-time quantitative PCR assay was performed in triplicate for IL-10 and IL-12 and G3PDH was used as an endogenous standard. All reactions were run as single-plex, and the relative gene expression was quantified by use of the $2^{-\Delta\Delta Ct}$ method. Amplification of 2 µl of cDNA was performed in a 25 µl PCR containing 900 nmol of each primer, 250 nmol of TaqMan probe and 12.5 µl of TaqMan® Gene Expression Master Mix[x]. Amplification and detection were performed using a 7500 Real Time PCR system[y]. The samples were amplified in a combined thermocycler-fluorometer for 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Each sample was assayed in triplicate, and the mean value was used for comparison. To account for variation in the amount and quality of starting material, all the results were normalized to

G3PDH expression. The threshold cycle values for each gene were compared with their respective standard curves to generate a relative transcript level.

TNF-α assay

The cell culture supernatant samples were thawed and assayed as described in Chapter 2 using a commercially available TNF-α ELISA screening kit.

Statistical analysis

TNF-α concentrations were reported as mean ± SD. Differences of gene expression between treatment groups and treatment time were evaluated by use of mixed-model repeated measures ANOVA. Pair-wise comparisons were made on significant differences identified with ANOVA using Tukey's post hoc test. A commercial statistical program[z] was used to perform analysis. Relative gene expression data were presented as $2^{-\Delta\Delta CT}$ and confidence intervals were reported. Pearson's correlation analysis was performed to evaluated correlation between TNF-α concentrations and mRNA expression of IL-10 and IL-12. Values of $P < 0.05$ were considered significant.

Results

TNF-α concentration

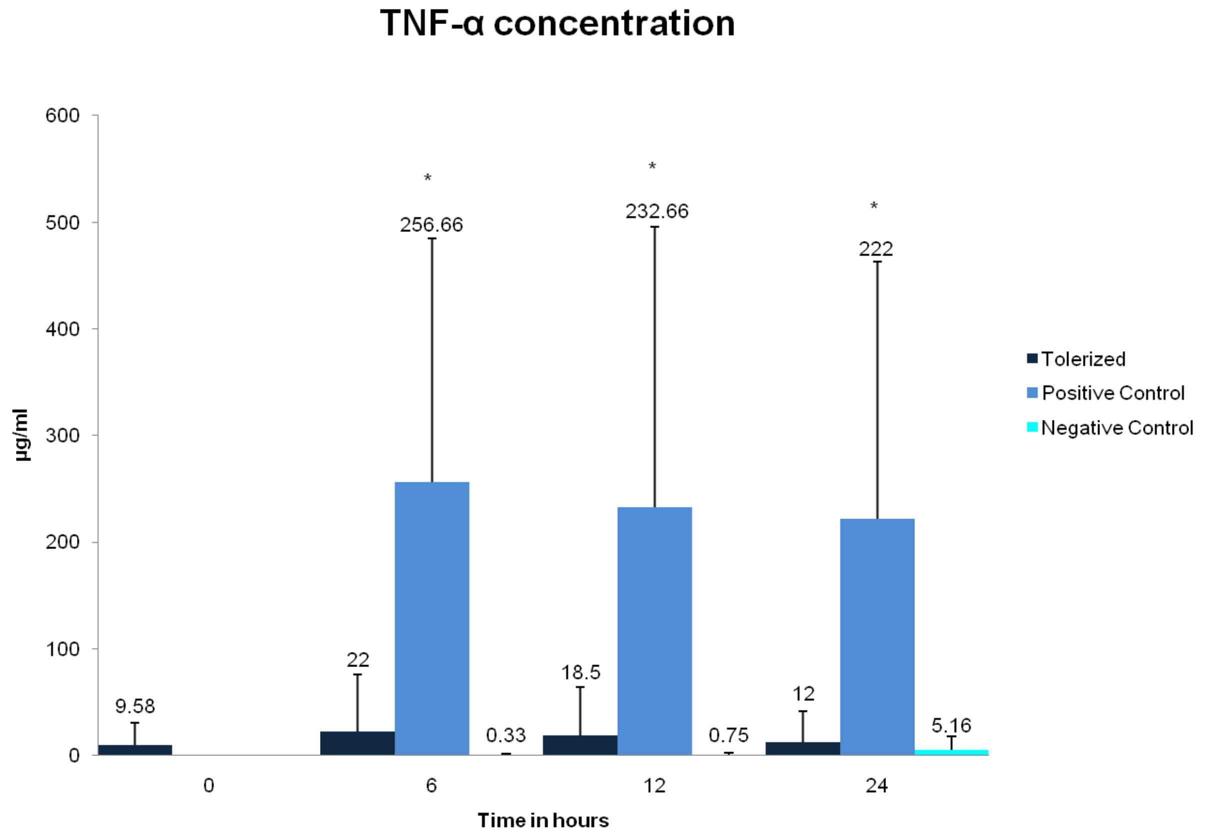

Figure 8. TNF-α concentration for negative control cells, positive control cells and tolerized cells. Data presented as mean ± SD. Numbers represent the mean TNF-α concentration for each group at the different time points. Asterisk (*) denotes statistically significant difference ($P < 0.0001$) between positive control cells and tolerized cells. Negative control cells did not produce any TNF-α at time 0. Positive control cells did not produce any TNF-α at time 0.

TNF-α was produced in minimal amounts by negative control cells after 6, 12 and 24 hours of cell culture. The positive control cells and tolerized cells expressed TNF-α after endotoxin exposure. There was a significant difference ($P < 0.0001$) in TNF-α secretion between positive control cells and tolerized cells. Endotoxin tolerance was induced in all tolerized cells that were

exposed to the 2-step endotoxin challenge. TNF-α secretion was decreased by greater than 85% in tolerized cells compared to positive control cells.

Gene expression of IL-10 and IL-12

A 2.4-fold increase in IL-10 expression was identified in tolerized cells when compared to negative and positive control cells at time 0 (Fig. 9). No statistically significant difference in IL-10 expression was observed between positive control cells and tolerized cells at time 6, 12 and 24, but tolerized cells showed decreased IL-10 expression when compared with positive control cells at time 6 and 12 (Fig.9). Negative control cells produced significantly less IL-10 at time 6, 12 and 24 when compared to positive control cells and tolerized cells. Significant effects of time ($P<0.0001$) and treatment ($P<0.0001$) were identified. When time and treatment effects were considered significant differences ($P<0.0273$) were observed between treatment groups within the 24 hour period. Our hypothesis regarding the mRNA expression of IL-10 was not confirmed. The mRNA expression of IL-10 from tolerized cells did not statistically differ from that of positive control cells at time 6, 12 and 24. A statistically significant difference between tolerized cells and positive control cells was only identified at time 0 when positive control cells had not been exposed to endotoxin yet.

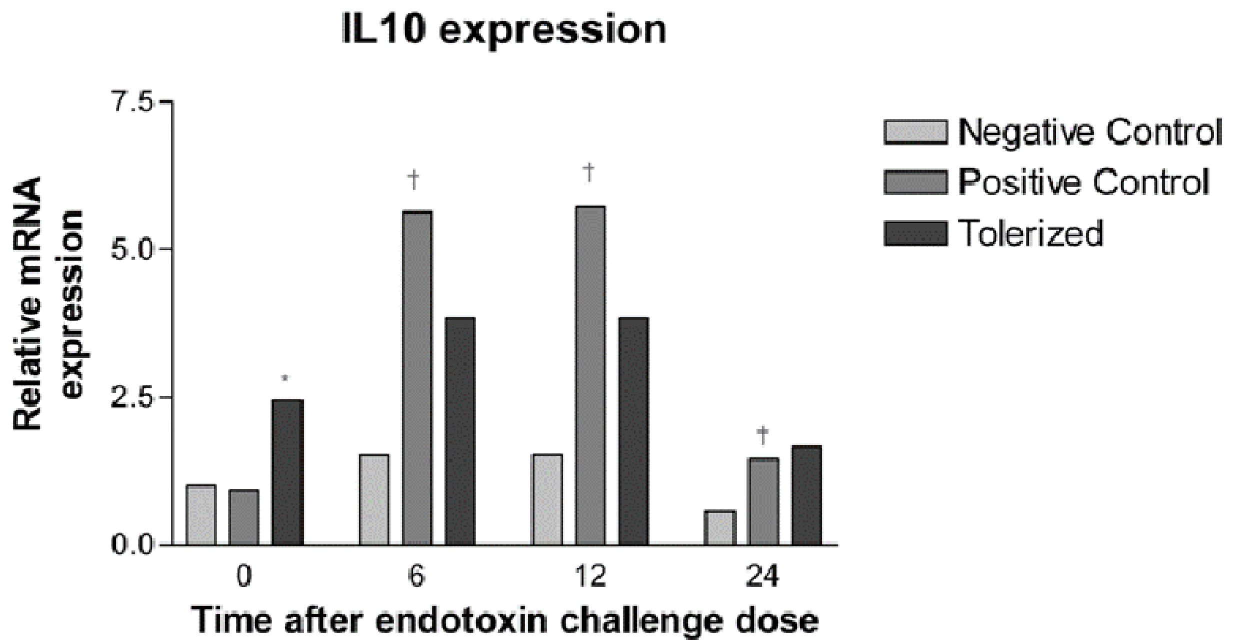

Figure 9. Mean relative expression of IL-10 of equine PBMCs after endotoxin exposure determined for a 24 hour period. Asterisk (*) denotes difference (P<0.025) between tolerized cells and negative control/positive control cells at time 0. Dagger (†) denotes difference (P<0.025) between negative control cells and positive control/tolerized cells at time 6, 12 and 24. Negative control cells at time 0 are used as baseline value with relative expression of 1.

Table 1. Relative gene expression 95% confidence interval for IL-10 in equine PBMCs over a 24 hour period.

Time	Negative Control Cells		Positive Control Cells		Tolerized Cells	
	95% Lower	95% Upper	95% Lower	95% Upper	95% Lower	95% Upper
0	0.184327334	0.904608	0.482941953	2.370095143	1.182687	5.804177
6	0.174527903	0.896944	0.118919277	0.611156583	0.300564	1.544676
12	0.175752384	0.903236	0.117853042	0.605676933	0.295794	1.520161
24	0.149011447	0.765808	0.17058665	0.876688432	0.504981	2.595228

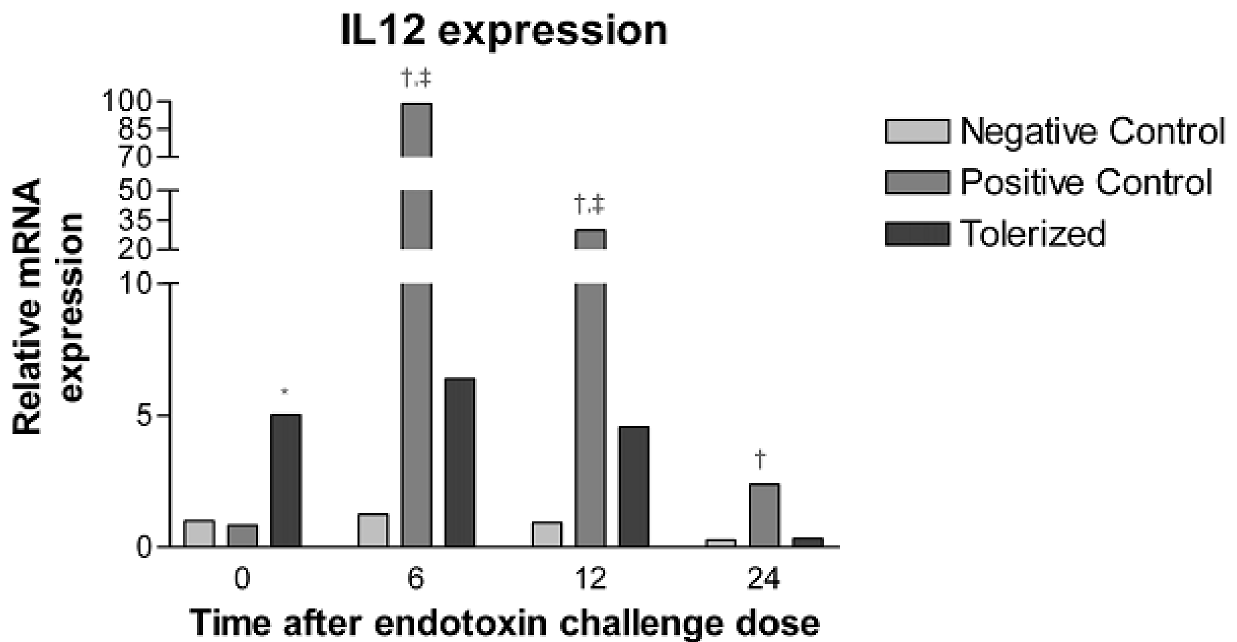

Figure 10. Mean relative expression of IL-12 of equine PBMCs after endotoxin exposure determined for a 24 hour period. Asterisk (*) denotes difference (P<0.0002) between tolerized cells and negative control/positive control cells at time 0. Dagger (†) denotes difference (P<0.0001) between positive control cells and negative control/tolerized cells at time 6, 12 and 24. Dagger (‡) denotes difference (P<0.0005) between negative control cells and tolerized cells at time 6 and 12. Negative control cells at time 0 are used as baseline value with relative expression of 1.

Table 2. Relative gene expression 95% confidence interval for IL-12 in equine PBMCs over a 24 hour period.

Time	Negative Control Cells		Positive Control Cells		Tolerized Cells	
	95% Lower	95% Upper	95% Lower	95% Upper	95% Lower	95% Upper
0	0.077989519	0.507322075	0.0467685401	3.042295056	2.351222949	15.29471296
6	0.074594438	0.512308122	0.004825063	0.033138112	0.024682215	0.169515308
12	0.078176729	0.536910985	0.011938825	0.081994815	0.058273581	0.400217891
24	0.314765788	2.161784085	0.04388319	0.301385939	0.053198364	0.365361741

A 5-fold increase in IL-12 expression was identified in tolerized cells when compared to negative and positive control cells at time 0 (Fig. 10). At time 6, 12 and 24 tolerized cells produced significantly less IL-12 than positive control cells ($P<0.0001$). A 15-fold decrease in IL-12 expression in tolerized cells compared with positive control cells was identified at time 6. A 6.6-fold decrease in IL-12 expression in tolerized cells compared with positive control cells was identified at time 12. Negative control cells produced significantly less IL-12 at time 6, 12 and 24 when compared to positive control cells ($P<0.0001$) and at time 6 and 12 when compared to tolerized cells ($P<0.0005$). Significant effects of time ($P<0.0001$) and treatment ($P<0.0001$) were identified. When time and treatment effects were considered significant differences ($P<0.0001$) were observed between treatment groups within the 24 hour period. Our hypothesis regarding the mRNA expression of IL-12 was confirmed. The mRNA expression of IL-12 from tolerized cells differed significantly from that of positive control cells at all time points.

Correlation between TNF-α concentration and mRNA expression of IL-10 and IL-12

TNF-α concentrations did not correlate with mRNA expression of IL-10 ($r = -0.115$, $P = 0.337$) (Fig. 11). A weak inverse correlation of TNF-α concentrations with mRNA expression of IL-12 was identified ($r = -0.324$, $P = 0.005$) (Fig. 12).

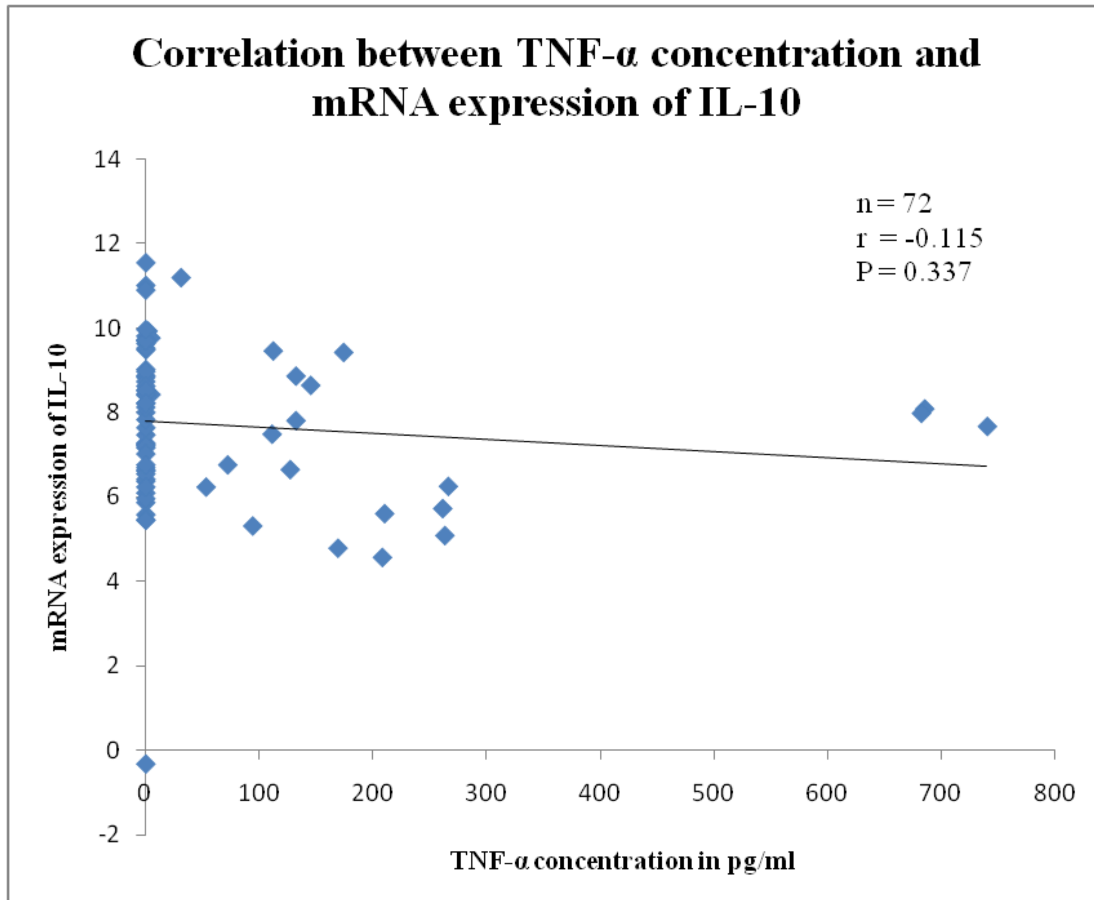

Figure 11. Correlation between TNF-α concentrations and mRNA expression of IL-10 in all three groups of cells. Pearson's correlation coefficient $r = -0.115$ with $P = 0.337$.

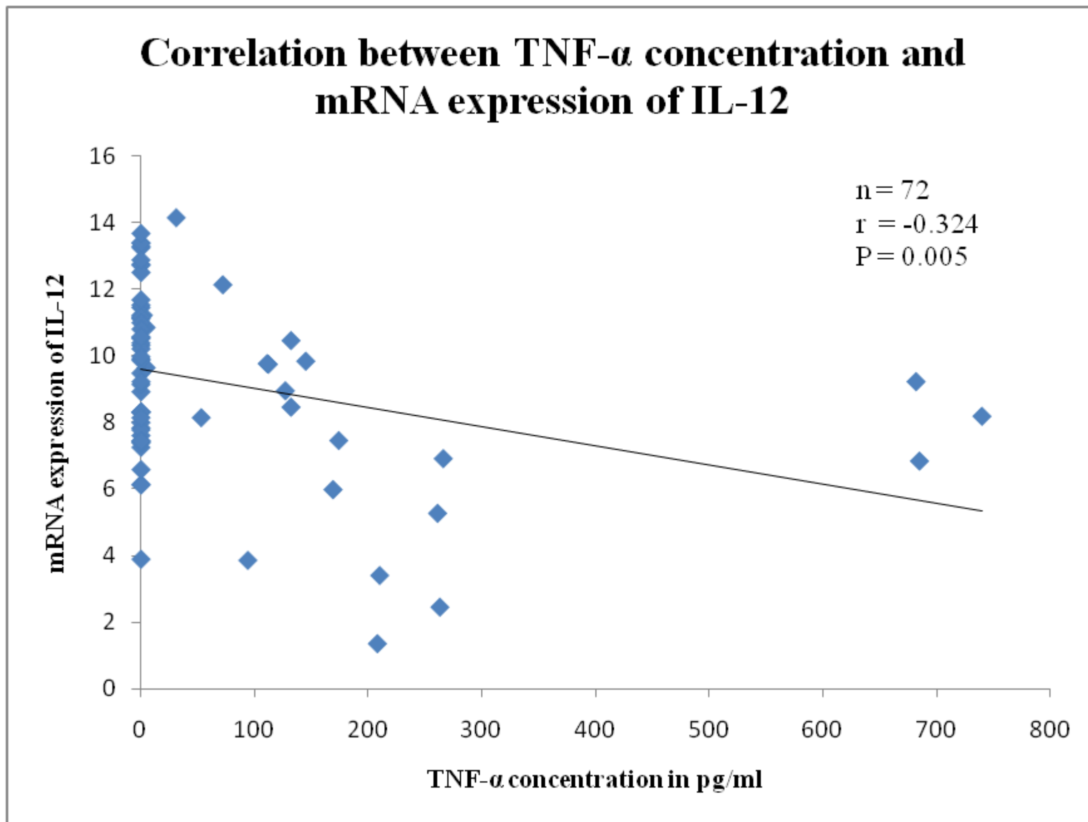

Figure 12. Correlation between TNF-α concentrations and mRNA expression of IL-12 in all three groups of cells. Pearson's correlation coefficient r = -0.324 with P = 0.005.

Chapter 4. Discussion

This study demonstrated that *in vitro* ET can be induced in equine PBMCs. A protocol for successful induction of ET *in vitro* was identified and was successfully repeated. ET was previously induced *in vivo* in horses and other species (Allen *et al.* 1996; Berg *et al.* 1995; Sanchez-Cantu *et al.* 1989). *In vitro* ET was induced in PBMCs from humans, mice, and pigs (Cagiola *et al.* 2006; de Waal Malefyt *et al.* 1991; Karp *et al.* 1998; Medvedev *et al.* 2000; Wysocka *et al.* 1995). This study provides an *in vitro* model for ET which can be used for further studies in equine PBMCs for investigation of cytokine characteristics in ET in horses.

TNF-α was used as a marker of endotoxemia and endotoxin tolerance and proved to be a reliable indicator of ET in this experiment. Quantification of TNF-α concentration in cell culture supernatant was an easy and time-efficient procedure. ET was defined as a reduction of TNF-α by equal to or greater than 75% compared to non-tolerized cells. In previous studies, TNF-α has been used as a phenotypic marker of ET and its production was decreased by 98-100% in most studies (Allen *et al.* 1996; Peck *et al.* 2004). TNF-α production peaks at 90-120 minutes after LPS exposure and was measured at that time in referenced studies (Allen *et al.* 1996; Peck *et al.* 2004). In our model, TNF-α concentrations were measured prior to and 6, 12 and 24 hours after LPS exposure. Therefore, it is likely that the TNF-α peak was missed, and we are unsure of the magnitude of TNF-α suppression at the peak. Our time points for sample collection were chosen to optimally assess the expression of other cytokines (IL-10 and IL-12) at the same time. These cytokines have been reported to reach their peak within 2 to 6 hours after LPS exposure (Beutler *et al.* 1986; Durez *et al.* 1993; van der Poll *et al.* 1994). While the magnitude of TNF-α suppression at the likely peak response was not determined, it is clear that tolerance was induced, and it is unlikely that this impacted the overall conclusions of the study.

At time 0 the IL-10 expression of tolerized cells was increased compared to negative and positive control cells because these cells had been exposed to endotoxin. Previously it has been described that IL-10 expression is increased after LPS exposure (de Waal Malefyt *et al.* 1991; Flohe *et al.* 1999; Marchant 1994; van der Poll *et al.* 1994). This increased IL-10 production is endotoxin-dose dependent (de Waal Malefyt *et al.* 1991) and is induced by pro-inflammatory cytokines including IL-1, IL-6 and TNF-α (van der Poll *et al.* 1994; Wanidworanun and Strober 1993). Enhanced production of IL-10 in endotoxemia and sepsis likely represents an attempt of the host to counteract excessive activity of pro-inflammatory cytokines (van der Poll *et al.* 1994). IL-10 is a potent inhibitor of LPS-induced TNF-α production *in vivo* and *in vitro* (de Waal Malefyt *et al.* 1991; Fiorentino *et al.* 1991; Hawkins *et al.* 1998) in dose-dependent fashion and has been shown to protect mice from endotoxin shock (Gerard *et al.* 1993; Marchant 1994). IL-10 induces the shift from pro- to anti-inflammatory cytokines (de Waal Malefyt and Moore 1998). IL-10 expression remained increased compared to negative control cells once ET was induced and slightly decreased compared to positive control cells. A continued expression of IL-10 was observed during the 24 hour period. This up-regulated IL-10 expression was not statistically significantly different from IL-10 expression in positive control cells. The upregulation of IL-10 expression prior to the LPS challenge dose and its continued expression thereafter suggest that these levels were sufficient in order to induce ET and to regulate a well-balanced Th1 and Th2 response in our model. Hawkins *et al.* suggested that IL-10 has potential as a novel anti-inflammatory agent for clinical application in horses since it suppressed the expression of pro-inflammatory cytokines in their study (Hawkins *et al.* 1998). Our results support this suggestion but further studies are needed to evaluate the benefit of IL-10 *in vivo*.

LPS-induced IL-10 expression by monocytes *in vitro* occurs 24 to 48 hours after LPS exposure (de Waal Malefyt *et al.* 1991), but IL-10 serum levels *in vivo* were already raised 3-6 hours after endotoxin challenge (Durez *et al.* 1993; van der Poll *et al.* 1994) indicating that other factors or cells may contribute to IL-10 production *in vivo*. Therefore, it is possible that we missed the peak IL-10 expression in our study since we did not collect any samples after 24 hours of LPS exposure.

IL-12 expression increases rapidly (within 3 hours) after LPS exposure (Karp *et al.* 1998; Sutterwala *et al.* 1997; van der Poll *et al.* 1997; Wysocka *et al.* 1995). Once ET was induced IL-12 expression was decreased (Karp *et al.* 1998; Wysocka *et al.* 1995). Our study confirmed these results. IL-12 expression was strongly up-regulated after LPS exposure and then decreased progressively. Low IL-12 increases the susceptibility to infection by intracellular bacteria (Sutterwala *et al.* 1997) but overproduction of IL-12 can be detrimental and may lead to exacerbated disease. Therefore, IL-12 expression needs to be tightly regulated in order to provide a well-balanced Th1 and Th2 response.

Future studies should include *in vitro* experiments of ET in equine PBMCs with the addition of IL-10 antibodies in order to evaluate its importance for the development of ET in equine PBMCs. Addition of recombinant IL-10 in models of endotoxemia should also be examined in order to assess the utility of IL-10 as a future therapeutic in equine endotoxemia. TGF-β is another potent anti-inflammatory cytokine which should be evaluated in equine endotoxemia and ET in order to characterize its importance for the development of ET. Ligation of macrophage receptors has been evaluated in rodents and represents a possible therapeutic for treatment of endotoxemia/induction of ET. The utility of this procedure should be assessed in equine PBMCs.

Conclusion

An *in vitro* model for induction of ET in equine PBMCs was successfully developed. An increased expression of IL-12 was associated with endotoxin exposure. IL-12 expression is suppressed in ET in equine PBMCs. IL-10 expression was up-regulated after endotoxin exposure and continued up-regulated expression of IL-10 was observed in endotoxin tolerant equine PBMCs. Further studies are required to assess the importance of IL-10 for development of ET in equine PBMCs.

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[a] BD Vacutainer Lithium Heparin, BD Biosciences, Franklin Lakes, NJ

[b] RPMI 1640 medium, Hepes modification, Sigma-Aldrich, St. Louis, MO

[c] Lymphoprep™, Accurate Chemical & Scientific Corp., Westbury, NY

[d] 15ml conical, polypropylene tubes, BD Labware, Franklin Lakes, NJ

[e] Fetal Bovine Serum, Sigma-Aldrich, St. Louis, MO

[f] Penicillin-streptomycin solution, Sigma-Aldrich, St. Louis, MO

[g] L-glutamine, Sigma-Aldrich, St. Louis, MO

[h] Hemocytometer, Hausser Scientific, Horsham, PA

[I] Trypan blue, Sigma-Aldrich, St. Louis, MO

[k] Lipopolysacchararides from *Escherichia coli* O55:B5, Sigma-Aldrich, St. Louis, MO

[l] Equine TNF-α screening set, Thermo Scoentific, Rockford, IL

[m] Dulbecco's PBS, HyClone®, HyClone Laboratories, Logan, Utah

[n] Tween®20, Acros Organics, Morris Plains, NJ

[o] BIO-RAD Model 550, Bio-Rad Laboratories, Hercules, CA

[p] SAS, SAS Institute, Cary, NC

[q] Heparin sodium, APP Pharmaceuticals, Schaumburg, IL

[r] Fenwal® transfer pack container, Baxter Healthcare Corporation, Deerfield, IL

[s] Protease inhibitor, Sigma-Aldrich, St. Louis, MO

[t] RNA isolation kit (RNeasy®), Qiagen Sciences, MD

[u] RNase-free DNase, Qiagen GmbH, Hilden, Germany

[v] Agarose BP160-100, Fisher Scientific, Fair Lawn, NJ

[w] High Capacity cDNA Reverse transcription kit, Applied Biosystems, Foster City, CA

[x] TaqMan® Gene Expression Master Mix, Applied Biosystems, Foster City, CA

[y] 7500 Real-Time PCR System, Applied Biosystems, Foster City, CA

[z] SAS PROC GLIMMIX, and JMP, SAS Institute Inc., Cary, NC

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<u>APPENDIX A</u>

Sequence information for primers and MGB probes for real time PCR analysis.

Gene of Interest	Forward Primer	Reverse Primer
Interleukin-10	GTCGGAGATGATCCAGTTTTACCT	AGTTCACGTGCTCCTTGATGTC
Interleukin-12p40	TGCTGTTCACAAGCTCAAGTATGA	GGGTGGGTCTGGTTTGATGA
G3PDH	GGTGGAGCCAAAAGGGTCAT	TTCACGCCCATCACAAACAT

Gene of Interest	Probe
Interleukin-10	TGCCCCAGGCTGAGAACCACG
Interleukin-12p40	CTACACCAGCGGCTTCTTCATCAGGG
G3PDH	TCTCTGCTCCTTCTGCTGATGCCCC